

PROTEIN FORMING COMPLEX WITH c-Jun PROTEIN, NUCLEIC ACID
ENCODING THE SAME AND METHOD OF USING THE SAME

Technical Field

[0001] The present invention relates to proteins that interact with c-Jun, nucleic acids encoding them and inhibitors utilizing them as well as methods for detecting an interaction and screening methods.

[0002] At present, genomic nucleotide sequences of various organisms are going to be elucidated. In researches of genomic sequences, there are expected, as post-sequencing researches of the second act, researches of analyzing meanings of the elucidated genomic information, i.e., structural and functional analyses of genes and proteins (Non-patent documents 1 and 2), analyses of protein/protein and protein/nucleic acid interactions (Non-patent documents 3 and 4) and so forth.

[0003] On the basis of analyses of networks of interactions between protein and protein, protein and nucleic acid and so forth in post-sequencing genomic functional analyses utilizing such techniques as described above, it is expected to create drugs and so forth standing on discoveries of novel functions of known proteins or important biological enzymes such as novel proteins that have been unknown so far.

[0004] As methods for detecting interactions between proteins, the immunoprecipitation (Non-patent document 5), pull-down assay based on a GST fusion protein (Non-patent document 6), TAP method (Non-patent document 7), yeast two-hybrid method (Non-patent document 8) and so forth are known so far. Further, as methods for comprehensive analysis of interactions between proteins in the post-sequencing genomic functional analyses utilizing the "assignment of gene (genotype) and protein (phenotype)" born as a tool of the evolutionary molecular engineering, there are the *in vitro* virus method (Non-patent documents 9

and 10, Patent documents 1 and 2), STABLE method (Non-patent document 11), phage display method (Non-patent document 12), ribosome display method (Non-patent document 13, Patent document 3), mRNA-peptide fusion method (mRNA display method, Non-patent document 14), and so forth.

[0005] Furthermore, the surface plasmon resonance method, fluorescence resonance energy transfer method, fluorescence depolarization method, evanescent-field imaging method, fluorescence correlation spectroscopy, fluorescent imaging method, enzyme linked immunosorbent assay and so forth are also known. Moreover, methods of modifying C-terminals of proteins in a translation system utilizing a nucleic acid derivatives such as puromycin have been previously proposed (Patent documents 4 and 5). These methods have advantages that functions of proteins are more unlikely to be damaged compared with the conventional chemical modification methods and the fluorescent protein fusion methods.

[0006] In the field of life science, sequence analysis of the human genome was completed, and genome researches rush into functional analyses of genes of the post-genome age. Thus, innovative drug creation based on comprehensive genomic functional analyses and so forth are expected. There is desired a technique that enables comprehensive analysis of genes and proteins, which have been independently studied so far, for example, a technique of analyzing various cofactors of transcription control factors as target proteins of drug creation at once and so forth. As a transcription control factor, the c-Fos/c-Jun proteins are known well.

[0007] The c-fos gene was isolated as a human homolog of which amino acid sequence indicates more than 80% identity or more with an oncogene, v-jun which is possessed by avian sarcoma virus 17 (the v-jun afterward became known to be derived from chicken genome and be conserved among the many species) (Non-patent document 15). The c-jun is a transcription control factor detected in many cytomas as a

typical immediate early gene in connection with a proliferataion stimulus. As known falimy genes, junB and junC are known. Generation of a null-mutated mouse by gene targeting and analysis conducted by Hilberg et al. confirmed that c-Jun-lacking mice exhibit impaired hepatogenesis and embryonically lethal. This suggested that an essential involvement of the c-jun gene in hepatogenesis (Non-patent document 16).

[0008] It is known that c-Jun has a trans-activation domain controlling transcription, at its N terminal, and a bZIP domain forming a homo/hetero dimer and binding a DNA element, at its C terminal (Non-patent document 17). Recently, screening by the two-hybrid method has been revealed that BAF60a, a subunit of the SWI/SNF chromatin remodeling complex interacts with the c-Fos/c-Jun complex (Non-patent document 18), and it is becoming known that the gene expression regulation by c-Jun is a complicated mechanism through structual change of chromatin, not a simple transcription model. Because there are many factors interacting with c-Jun, there is a high possibility that c-Jun plays an important role as a hub of protein-protein interactions in the maintenance of the complicated mechanism. The regulation of chromatin is an esstial mechanism involved in development and differentiation and is associated with various diseases including cancer. Thus, comprehensive analyses of factors interacting with c-Jun will make an important impact on not only basic study but also drug creation. However, such a thorough 1:1 molecule analysis technique as the two-hybrid method takes enormous time and labor and, therefore, is not practical.

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Disclosure of the Invention

[0009] An object of the present invention is to provide a complex that interacts with the c-Jun protein, which is well known as a transcription control factor, as a target protein.

[0010] The inventors of the present invention conducted comprehensive analysis of transcription control factor complexes in a mouse brain cDNA library with c-Jun as a bait by using two of techniques, the cotranslation screening of *in vitro* virus (IVV) and the C-terminal labeling method (U.S. Patent No. 6,228,994, WO02/48347) named puromycin technologies, which have been researched on the basis of the aforementioned *in vitro* virus method as a method for comprehensive analysis as a one-to-multiple molecule-analysis method replacing the method, and thereby attempted to analyze proteins unknown so far, proteins known so far, but unknown to form a complex with the c-Jun protein, and so forth. The expression of "a protein that form a complex" used herein refers to a protein that directly or indirectly interacts with the c-Jun protein.

[0011] A further object of the present invention is to provide a protein that interacts with c-Jun and an inhibitor utilizing it, as well as a method for detecting an interaction and method for screening.

[0012] The inventors of the present invention found

novel proteins that interact with c-Jun by the cotranslation screening using IVV, also found that certain known proteins interacted with c-Jun, and accomplished the present invention. The present invention thus provides the followings.

[0013] (1) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 1 to 69,

(b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 1 to 69 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0014] (2) The inhibitor according to (2), wherein the protein as the active ingredient comprises any one of the amino acid sequences of SEQ ID NOS: 1 to 69.

[0015] (3) The inhibitor according to (2), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

(a) a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 126 to 199,

(b) a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 126 to 199 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0016] (4) The inhibitor according to (3), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 126 to 199.

[0017] (5) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

(a) a protein comprising any one of the amino acid

sequences of SEQ ID NOS: 1 to 69,

(b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 1 to 69 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 126 to 199,

(b') a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 126 to 199 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0018] (6) The method according to (5), wherein the protein comprises any one of the amino acid sequences of SEQ ID NOS: 1 to 69.

[0019] (7) The method according to (5), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 126 to 199.

[0020] (8) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (5) to (7) and the step of selecting a prey for which an interaction is detected.

[0021] (9) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 70 to 87,

(b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 70 to 87 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0022] (10) The inhibitor according to (9), wherein the protein as the active ingredient comprises any one of the amino acid sequences of SEQ ID NOS: 70 to 87.

[0023] (11) The inhibitor according to (9), wherein the protein is a protein translated from a nucleic acid of

the following (a) or (b):

(a) a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 200 to 217,

(b) a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 200 to 217 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0024] (12) The inhibitor according to (11), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 200 to 217.

[0025] (13) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

(a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 70 to 87,

(b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 70 to 87 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 200 to 217,

(b') a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 200 to 217 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0026] (14) The method according to (13), wherein the protein comprises any one of the amino acid sequences of SEQ ID NOS: 70 to 87.

[0027] (15) The method according to (13), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 200 to 217.

[0028] (16) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the

method according to any one of (13) to (15) and the step of selecting a prey for which an interaction is detected.

[0029] (17) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 88 to 94,

(b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 88 to 94 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0030] (18) The inhibitor according to (17), wherein the protein as the active ingredient comprises any one of the amino acid sequences of SEQ ID NOS: 88 to 94.

[0031] (19) The inhibitor according to (17), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

(a) a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 218 to 224,

(b) a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 218 to 224 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0032] (20) The inhibitor according to (19), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 218 to 224.

[0033] (21) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

(a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 88 to 94,

(b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 88 to 94 including deletion,

substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 218 to 224,

(b') a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 218 to 224 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0034] (22) The method according to (21), wherein the protein comprises any one of the amino acid sequences of SEQ ID NOS: 88 to 94.

[0035] (23) The method according to (21), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 218 to 224.

[0036] (24) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (21) to (23) and the step of selecting a prey for which an interaction is detected.

[0037] (25) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 95 to 99,

(b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 95 to 99 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0038] (26) The inhibitor according to (25), wherein the protein as the active ingredient comprises any one of the amino acid sequences of SEQ ID NOS: 95 to 99.

[0039] (27) The inhibitor according to (25), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

(a) a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 225 to 229,

(b) a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 225 to 229 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0040] (28) The inhibitor according to (27), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 225 to 229.

[0041] (29) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

(a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 95 to 99,

(b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 95 to 99 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 225 to 229,

(b') a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 225 to 229 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0042] (30) The method according to (29), wherein the protein comprises any one of the amino acid sequences of SEQ ID NOS: 95 to 99.

[0043] (31) The method according to (29), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 225 to 229.

[0044] (32) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (29) to (31) and the step of selecting a prey for which an interaction is detected.

[0045] (33) An inhibitor for an interaction between a

protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 100 to 104,

(b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 100 to 104 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0046] (34) The inhibitor according to (33), wherein the protein as the active ingredient comprises any one of the amino acid sequences of SEQ ID NOS: 100 to 104.

[0047] (35) The inhibitor according to (33), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

(a) a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 230 to 234,

(b) a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 230 to 234 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0048] (36) The inhibitor according to (3), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 230 to 234.

[0049] (37) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

(a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 100 to 104,

(b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 100 to 104 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising any one of the nucleotide

sequences of SEQ ID NOS: 230 to 234,

(b') a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 230 to 234 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0050] (38) The method according to (37), wherein the protein comprises any one of the amino acid sequences of SEQ ID NOS: 100 to 104.

[0051] (39) The method according to (37), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 230 to 234.

[0052] (40) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (37) to (39) and the step of selecting a prey for which an interaction is detected.

[0053] (41) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 105 to 108,

(b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 105 to 108 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0054] (42) The inhibitor according to (41), wherein the protein as the active ingredient comprises any one of the amino acid sequences of SEQ ID NOS: 105 to 108.

[0055] (43) The inhibitor according to (41), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

(a) a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 235 to 238,

(b) a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 235 to 238 under a stringent condition and encodes a

protein that interacts with the c-Jun protein.

[0056] (44) The inhibitor according to (43), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 235 to 238.

[0057] (45) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

(a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 105 to 108,

(b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 105 to 108 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 235 to 238,

(b') a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 235 to 238 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0058] (46) The method according to (45), wherein the protein comprises any one of the amino acid sequences of SEQ ID NOS: 105 to 108.

[0059] (47) The method according to (45), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 235 to 238.

[0060] (48) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (45) to (47) and the step of selecting a prey for which an interaction is detected.

[0061] (49) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

- (a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 109 to 111,
- (b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 109 to 111 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0062] (50) The inhibitor according to (49), wherein the protein as the active ingredient comprises any one of the amino acid sequences of SEQ ID NOS: 109 to 111.

[0063] (51) The inhibitor according to (49), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

- (a) a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 239 to 241,
- (b) a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 239 to 241 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0064] (52) The inhibitor according to (51), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 239 to 241.

[0065] (53) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

- (a) a protein comprising any one of the amino acid sequences of SEQ ID NOS: 109 to 111,
- (b) a protein that comprises any one of the amino acid sequences of SEQ ID NOS: 109 to 111 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,
- (a') a nucleic acid comprising any one of the nucleotide sequences of SEQ ID NOS: 239 to 241,
- (b') a nucleic acid that hybridizes with a nucleic acid comprising any one of the nucleotide sequences of SEQ ID

NOS: 239 to 241 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0066] (54) The method according to (53), wherein the protein comprises any one of the amino acid sequences of SEQ ID NOS: 109 to 111.

[0067] (55) The method according to (53), wherein the nucleic acid comprises any one of the nucleotide sequences of SEQ ID NOS: 239 to 241.

[0068] (56) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (53) to (55) and the step of selecting a prey for which an interaction is detected.

[0069] (57) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising the amino acid sequence of SEQ ID NO: 112 or 113,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 112 or 113 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0070] (58) The inhibitor according to (57), wherein the protein as the active ingredient comprises the amino acid sequence of SEQ ID NO: 112 or 113.

[0071] (59) The inhibitor according to (57), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

(a) a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 242 or 243,

(b) a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 242 or 243 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0072] (60) The inhibitor according to (59), wherein the nucleic acid comprises the nucleotide sequence of SEQ

ID NO: 242 or 243.

[0073] (61) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

(a) a protein comprising the amino acid sequence of SEQ ID NO: 112 or 113,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 112 or 113 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 242 or 243,

(b') a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 242 or 243 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0074] (62) The method according to (61), wherein the protein comprises the amino acid sequence of SEQ ID NO: 112 or 113.

[0075] (63) The method according to (61), wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO: 242 or 243.

[0076] (64) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (61) to (63) and the step of selecting a prey for which an interaction is detected.

[0077] (65) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising the amino acid sequence of SEQ ID NO: 114 or 115,

(b) a protein that comprises the amino acid sequence of SEQ

ID NO: 114 or 115 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0078] (66) The inhibitor according to (65), wherein the protein as the active ingredient comprises the amino acid sequence of SEQ ID NO: 114 or 115.

[0079] (67) The inhibitor according to (65), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

(a) a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 244 or 245,

(b) a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 244 or 245 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0080] (68) The inhibitor according to (67), wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO: 244 or 245.

[0081] (69) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

(a) a protein comprising the amino acid sequence of SEQ ID NO: 114 or 115,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 114 or 115 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 244 or 245,

(b') a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 244 or 245 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0082] (70) The method according to (69), wherein the

protein comprises the amino acid sequence of SEQ ID NO: 114 or 115.

[0083] (71) The method according to (69), wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO: 244 or 245.

[0084] (72) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (69) to (71) and the step of selecting a prey for which an interaction is detected.

[0085] (73) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising the amino acid sequence of SEQ ID NO: 116 or 117,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 116 or 117 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0086] (74) The inhibitor according to (73), wherein the protein as the active ingredient comprises the amino acid sequence of SEQ ID NO: 116 or 117.

[0087] (75) The inhibitor according to (73), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

(a) a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 246 or 247,

(b) a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 246 or 247 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0088] (76) The inhibitor according to (75), wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO: 246 or 247.

[0089] (77) A method for detecting an interaction between a bait and a prey, which comprises bringing the

bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

(a) a protein comprising the amino acid sequence of SEQ ID NO: 116 or 117,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 116 or 117 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 246 or 247,

(b') a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 246 or 247 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0090] (78) The method according to (77), wherein the protein comprises the amino acid sequence of SEQ ID NO: 116 or 117.

[0091] (79) The method according to (77), wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO: 246 or 247.

[0092] (80) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (77) to (79) and the step of selecting a prey for which an interaction is detected.

[0093] (81) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising the amino acid sequence of SEQ ID NO: 118 or 119,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 118 or 119 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0094] (82) The inhibitor according to (81), wherein the protein as the active ingredient comprises the amino acid sequence of SEQ ID NO: 118 or 119.

[0095] (83) The inhibitor according to (81), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

(a) a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 248 or 249,

(b) a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 248 or 249 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0096] (84) The inhibitor according to (83), wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO: 248 or 249.

[0097] (85) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

(a) a protein comprising the amino acid sequence of SEQ ID NO: 118 or 119,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 118 or 119 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 248 or 249,

(b') a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 248 or 249 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0098] (86) The method according to (85), wherein the protein comprises the amino acid sequence of SEQ ID NO: 118 or 119.

[0099] (87) The method according to (85), wherein the

nucleic acid comprises the nucleotide sequence of SEQ ID NO: 248 or 249.

[0100] (88) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (85) to (88) and the step of selecting a prey for which an interaction is detected.

[0101] (89) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising the amino acid sequence of SEQ ID NO: 120 or 121,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 120 or 121 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0102] (90) The inhibitor according to (89), wherein the protein as the active ingredient comprises the amino acid sequence of SEQ ID NO: 120 or 121.

[0103] (91) The inhibitor according to (89), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

(a) a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 250 or 251,

(b) a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 250 or 251 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0104] (92) The inhibitor according to (91), wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO: 250 or 251.

[0105] (93) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic

acid of the following (a') or (b')):

(a) a protein comprising the amino acid sequence of SEQ ID NO: 120 or 121,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 120 or 121 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 250 or 251,

(b') a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 250 or 251 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0106] (94) The method according to (93), wherein the protein comprises the amino acid sequence of SEQ ID NO: 120 or 121.

[0107] (95) The method according to (93), wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO: 250 or 251.

[0108] (96) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (93) to (95) and the step of selecting a prey for which an interaction is detected.

[0109] (97) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising the amino acid sequence of SEQ ID NO: 122 or 123,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 122 or 123 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0110] (98) The inhibitor according to (97), wherein the protein as the active ingredient comprises the amino acid sequence of SEQ ID NO: 122 or 123.

[0111] (99) The inhibitor according to (97), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

(a) a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 252 or 253,

(b) a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 252 or 253 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0112] (100) The inhibitor according to (99), wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO: 252 or 253.

[0113] (101) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

(a) a protein comprising the amino acid sequence of SEQ ID NO: 122 or 123,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 122 or 123 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 252 or 253,

(b') a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 252 or 253 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0114] (102) The method according to (101), wherein the protein comprises the amino acid sequence of SEQ ID NO: 122 or 123.

[0115] (103) The method according to (101), wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO: 252 or 253.

[0116] (104) A method for screening for a prey that

interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (101) to (103) and the step of selecting a prey for which an interaction is detected.

[0117] (105) An inhibitor for an interaction between a protein that interacts with a c-Jun protein and the c-Jun protein, which comprises a protein of the following (a) or (b) as an active ingredient:

(a) a protein comprising the amino acid sequence of SEQ ID NO: 124 or 125,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 124 or 125 including deletion, substitution or addition of one or several amino acid residues and interacts with the c-Jun protein.

[0118] (106) The inhibitor according to (105), wherein the protein as the active ingredient comprises the amino acid sequence of SEQ ID NO: 124 or 125.

[0119] (107) The inhibitor according to (105), wherein the protein is a protein translated from a nucleic acid of the following (a) or (b):

(a) a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 254 or 255,

(b) a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 254 or 255 under a stringent condition and encodes a protein that interacts with the c-Jun protein.

[0120] (108) The inhibitor according to (107), wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO: 254 or 255.

[0121] (109) A method for detecting an interaction between a bait and a prey, which comprises bringing the bait and the prey into contact and detecting a complex formed by the contact, wherein the bait is a protein of the following (a) or (b) or a protein translated from a nucleic acid of the following (a') or (b'):

(a) a protein comprising the amino acid sequence of SEQ ID NO: 124 or 125,

(b) a protein that comprises the amino acid sequence of SEQ ID NO: 124 or 125 including deletion, substitution or addition of one or several amino acid residues and interacts with a c-Jun protein,

(a') a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 254 or 255,

(b') a nucleic acid that hybridizes with a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 254 or 255 under a stringent condition and encodes a protein that interacts with a c-Jun protein.

[0122] (110) The method according to (109), wherein the protein comprises the amino acid sequence of SEQ ID NO: 124 or 125.

[0123] (111) The method according to (109), wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO: 254 or 255.

[0124] (112) A method for screening for a prey that interacts with a bait, which comprises the step of detecting an interaction between the bait and a prey by the method according to any one of (109) to (111) and the step of selecting a prey for which an interaction is detected.

Brief Explanation of the Drawings

[0125] [Fig. 1] It collectively shows information of amino acid sequence, gene sequence and so forth of the protein of the present invention. Each of numerals within the parentheses after the DNA sequence numbers indicates the number of amino acid sequence encoded by it. A number having a subnumber means that the DNA encodes the same amino acid sequence, but has a different nucleotide sequence.

[Fig. 2] It shows the outline of the cotranslation screening method using an IVV random library, which is a method for detecting the proteins and genes of the present invention and the nucleotide sequences thereof. An IVV random library of mouse brain and c-Jun as a bait were used to carry out cell-free cotranslation screening, and the

library after the screening was amplified by RT-PCR and then subjected to the cell-free cotranslation screening again with the bait. This procedure was repeated 3 times to detect the proteins and genes of the present invention and the nucleotide sequences thereof.

[Fig. 3] It shows the random priming library of IVV used for detection of the proteins and genes of the present invention and the nucleotide sequences thereof, and the outline of the method for producing it. Using an RNA library as a template and a random primer including a random sequence consisting of nine nucleotides and a specific sequence (tag2 sequence) in the random priming method, a library of single strand cDNAs (ssDNA) complementary to mRNAs is synthesized by reverse transcription (I). Only RNAs are decomposed in the double strands of cDNA and RNA with RNaseH, at the same time, DNAs complementary to cDNAs are synthesized with DNA polymerase I, and further a nick existing between the DNAs synthesized with DNA polymerase I is modified by using a DNA ligase to synthesized a double strand (dsDNA) library (II). The synthesized double-stranded cDNAs have a phosphate group at the 5' end only on the side synthesized with the DNA polymerase I, and therefore this is utilized to ligate an adaptor having a specific sequence (5' UTR = promoter + enhancer) using a DNA ligase to synthesize a ligated dsDNA library (III). PCR is carried out by utilizing the adaptor and the specific sequence of the random primer to prepare a cDNA library of assigning molecules having the sequences of a promoter and an enhancer on the 5' end side and an A tail on the 3' end side (IVV cDNA library) (IV). Then, the IVV cDNA library is transcribed to form an IVV RNA library (V), a spacer for preparing IVV is ligated (VI), and further it is translated in a cell-free translation system or the like to form a library of assigning molecules (VII).

[Fig. 4] It shows the result of the verification of the interactions of the proteins and genes of the present invention (electrophoretic photographs) and the nucleotide

sequences thereof.

A: It was confirmed by the C-terminal labeling method in a wheat cell-free translation system that the proteins of SEQ ID NOS: 18 (SNAP19), 76 (KINN), 93 (Kif5a), 99 (Eef1d), 102 (Nef3), 106 (Jip-c3.1), 110 (Jip-cl), 113 (EB2), 115 (Cspg6), 117 (Mapk8ip3), 119 (Jip-c3.2), 121 (GFAP), 123 (Jip-c8) and 125 (Kif5b) (Fig. 1) were expressed from the nucleic acid sequences of SEQ ID NOS: 143, 206, 223, 229, 232, 236, 240, 243, 245, 247, 249 and 253. Lanes 1 to 14: proteins of SEQ ID NOS: 18 (SNAP19), 76 (KINN), 93 (Kif5a), 99 (Eef1d), 102 (Nef3), 106 (Jip-c3.1), 110 (Jip-cl), 113 (EB2), 115 (Cspg6), 117 (Mapk8ip3), 119 (Jip-c3.2), 121 (GFAP), 123 (Jip-c8) and 125 (Kif5b).

B: As a verification experiment of the interactions of the obtained proteins and c-Jun, direct interactions with c-Jun were confirmed by pull-down using C-terminal labeled proteins having the amino acid sequences of the proteins of SEQ ID NOS: 18 (SNAP19), 76 (KINN), 93 (Kif5a), 106 (Jip-c3.1), 110 (Jip-cl), 113 (EB2), 115 (Cspg6), 117 (Mapk8ip3), 119 (Jip-c3.2) and 123 (Jip-c8) based on the nucleic acid sequences of SEQ ID NOS: 143, 206, 223, 236, 240, 243, 245 and 249. Group 1: SEQ ID NO: 18 (SNAP19), Group 2: 76 (KINN), Group 3: 93 (Kif5a), Group 6: 106 (Jip-c3.1), Group 7: 110 (Jip-cl), Group 8: 113 (EB2), Group 9: 115 (Cspg6), Group 10: 117 (Mapk8ip3), Group 11: 119 (Jip-c3.2) and Group 13: 123 (Jip-c8); a and b: with and without bait c-Jun (Lanes 1, 2 and 3: translation product, supernatant fraction and eluted fraction).

[Fig. 5] It shows the result of verification of the concentration rate and indirect interaction of the genes of the present invention. In order to confirm concentrations of 14 kinds of proteins of SEQ ID NOS: 18 (SNAP19), 76 (KINN), 93 (Kif5a), 99 (Eef1d), 102 (Nef3), 106 (Jip-c3.1), 110 (Jip-cl), 113 (EB2), 115 (Cspg6), 117 (Mapk8ip3), 119 (Jip-c3.2), 121 (GFAP), 123 (Jip-c8) and 125 (Kif5b), real-time PCR was performed by using the nucleic acid sequences of SEQ ID NO: 143, 206, 223, 229, 232, 236, 240, 243, 245,

247, 249 and 253.

[Fig. 6] It shows outlines of the primary screening and secondary screening in the analysis of interaction of IVV with substances or proteins using the proteins and nucleic acid sequences of the present invention. It is possible to detect interactions with the substances and proteins in the primary screening using the proteins and nucleic acid sequences of the present invention, and further analyze the details of the interaction in the secondary screening using FCCS, microarray or the like. Further, the proteins and nucleic acid sequences of the present invention can also be independently used for analysis of interaction with the substances or proteins using FCCS, microarray, or the like as IVV or C-terminal labeled proteins. Furthermore, it is also possible to apply them to the evolutionary molecular engineering using IVV of the proteins or nucleic acid sequences of the present invention, and utilize them to create functional proteins in the primary screening. In such a case, it is also possible to analyze details of interactions of the created functional proteins with a combination of the primary screening and secondary screening.

[Fig. 7] It shows configurations of a translation template (A) as well as a coding molecule (B) and spacer molecule (C), which are constituents of the template. The translation template consists of a coding portion derived from the coding molecule and a spacer portion derived from the spacer molecule. F1 and F2 represent a fluorescent dye.

[Fig. 8] shows configurations of a protein modified at the C-terminal (C-terminal labeled protein) (A), translation template of the present invention (B) and modification agent (C).

[Fig. 9] It shows outline of formation of a complex by cell-free cotranslation.

A: Both of bait and prey are translated in a cell-free translation system to interact with each other to form a complex in the cell-free translation system. The prey may

exist in a single number (I) or multiple number (II), and it may be a polypeptide itself obtainable by the translation in the cell-free translation system, or an assigning molecule (bound substance).

B: In the presence of the bait, the prey is translated in a cell-free translation system to interact with the bait and thereby form a complex in the cell-free translation system. The prey may exist in a single number (I) or multiple number (II), and it may be a polypeptide itself obtainable by the translation in the cell-free translation system, or an assigning molecule (bound substance).

[Fig. 10] It shows outline of formation of a complex by cell-free cotranslation using a complexed bait.

Both of bait as a part constituting the complexed bait and prey are translated in a cell-free translation system and interact to form a complex in the cell-free translation system. The prey may exist in a single number (I) or multiple number (II), and it may be a polypeptide itself obtainable by the translation in the cell-free translation system, or an assigning molecule (bound substance). Further, the complexed bait is not limited to the combination of a polypeptide translated in a cell-free translation system and DNA bait shown in the drawing, and it may be, for example; a combination of multiple or single polypeptide translated in a cell-free translation system and multiple or single bait coexisting in the cell-free translation system (e.g., DNA bait etc.), or the like.

[Fig. 11] It shows outline of the method of screening for a complex based on cell-free cotranslation.

By the step (1) of forming a complex on the basis of cell-free cotranslation as shown in Figs. 9 and 10, the step (2) of screening the prey of the complex, and the step (3) of analyzing the prey, the cell-free cotranslation and screening can be realized totally *in vitro*. If the prey is an assigning molecule, and it exists in a multiple number, the screening can be repeated again from the step (1) by reconstructing mRNA or DNA encoding the prey by RT-PCR or

PCR. Further, after analyzing the obtained prey, screening can be newly repeated from the step of (1) using the prey as a bait.

Best Mode for Carrying out the Invention

[0126] <1> Proteins of the present invention

In this specification, proteins found to interact with c-Jun, including novel proteins, are called "the proteins of the present invention" for convenience of explanation.

[0127] The first group of the proteins of the present invention consisting of proteins of which function is known but of which function of interacting with c-Jun is novel. These proteins are not any of proteins known to form a complex with c-Jun so far (Yurii Chinenov1 and Tom K Kerppola, *Oncogene* (2001) 20, 2438-2452). Specifically, as shown in Fig. 1, SNAP19(SNAPc5) corresponding to the amino acid sequence SEQ ID NOS: 1 to 69 is one of subunits of the SNAP complex binding to PSE, a promoter of snRNA transcribed by polII and pol III, thereby regulating transcription (Henry, R. W.; Mittal, V.; Ma, B.; Kobayashi, R.; Hernandez, N. *Genes Dev.* 12: 2664-2672, 1998. (PubMed ID : 9732265)). Kif5c corresponding to the amino acid sequence SEQ ID NOS: 70 to 87 is one molecule of the kinesin superfamily, which is a motor protein involved in substance transportation along with microtubule. It is known to be highly expressed in brain (Nagase, T.; Ishikawa, K.; Miyajima, N.; Tanaka, A.; Kotani, H.; Nomura, N.; Ohara, O. *DNA Res.* 5: 31-39, 1998. (PubMed ID : 9628581)). Kif5a corresponding to the amino acid sequence SEQ ID NOS: 88 to 94 is one molecule of the kinesin superfamily, which is a motor protein involved in substance transportation along with microtubule. It is known that its mutation results in hereditary spastic paraplesia (Niclas, J.; Navone, F.; Hom-Booher, N.; Vale, R. D. *Neuron* 12: 1059-1072, 1994. (PubMed ID : 7514426)). Eef1d corresponding to the amino acid sequence SEQ ID NOS: 95 to 99 is known as a protein

regulating elongation of translation and recently revealed that it is also a product of a gene associated with cancer and tumor (Joseph, P.; Lei, Y. X.; Whong, W. Z.; Ong, T. M. *J. Biol. Chem.* 277 : 6131-6136, 2002. (PubMed ID : 11711542)). Nef3 corresponding to the amino acid sequence SEQ ID NOS: 100 to 104 is one of three molecules constituting neuron-specific intermediate filaments and is a protein with a molecular weight of 125 kD (Levy, E.; Liem, R. K. H.; D'Eustachio, P.; Cowan, N. J. *Europ. J. Biochem.* 166: 71-77, 1987. (PubMed ID : 3036526)). EB2 corresponding to the amino acid sequence SEQ ID NOS: 112 to 113 is APC-binding protein EB2(Mapre3) and is one of the EB1 family known to interact with APC to cooperatively promote aggregation of microtubule (Su, L.-K.; Qi, Y. : Characterization of human MAPRE genes and their proteins. *Genomics* 71: 143-149, 2001). Cspg6 corresponding to the amino acid sequence SEQ ID NOS: 114 to 115 is one of subunits of cohesin complexes which play an important role in sister chromatid partition and a protein composed of a globular portion having ATPase activity and a rod portion (Sumara, I.; Vorlaufer, E.; Gieffers, C.; Peters, B. H.; Peters, J.-M. *J. Cell Biol.* 151: 749-761, 2000. (PubMed ID : 11076961)). Mapk8ip3 corresponding to the amino acid sequence SEQ ID NOS: 116 to 117 is one of scaffold proteins binding to JNK and considered to play an important role in the MAP kinase signaling cascade mediated by JNK (Dickens, M.; Davis, R. J. *Molec. Cell. Biol.* 20: 1030-1043, 2000. (PubMed ID : 10629060)). GFAP corresponding to the amino acid sequende SEQ ID NOS: 120 to 121 is one of constituents of intermediate filaments highly expressed in glial cells of the astrocyte lineage. It has a specific sequence at its N terminal and is considered to play a cell-specific role. It is a causal gene for a strange disease, Alexander disease (Bongcam-Rudloff, E.; Nister, M.; Betsholtz, C.; Wang, J.-L.; Stenman, G.; Huebner, K.; Croce, C. M.; Westermarck, B. *Cancer Res.* 51: 1553-1560, 1991. (PubMed ID : 1847665)). Kif5b corresponding to the amino acid

sequence SEQ ID NOS: 124 to 125 is one of the kinesin superfamily, which is a motor protein involved in substance transportation along with microtubule. It is involved in organelle transportation and abnormal distribution of mitochondria occurs in its KO mouse (Tanaka, Y.; Kanai, Y.; Okada, Y.; Nonaka, S.; Takeda, S.; Harada, A.; Hirokawa, N. Cell 93: 1147-1158, 1998. PubMed ID : 9657148), Kamal, A.; Almenar-Queralt, A.; LeBlanc, J. F.; Roberts, E. A.; Goldstein, L. S. B. Nature 414: 643-648, 2001. (PubMed ID : 11740561)).

[0128] Among these proteins, those having no leucine zipper are Nef3, EB2, GFAP and Kif5b. Those indirectly interacting with c-Jun are Nef3 and GFAP. All the others, SNAP19, Kif5c, Kif5a, Eef1d, Cspg6 and Mapk8ip3 have the leucine zipper and form a complex with c-Jun.

[0129] The second group of the proteins of the present invention consisting of proteins of which function is unknown and of which function of interacting with c-Jun is newly found. Specifically, these are Jip-c3.1 corresponding to the amino acid sequence SEQ ID NOS: 105 to 108, Jip-c1 corresponding to the amino acid sequence SEQ ID NOS: 109 to 111, Jip-c3.2 corresponding to the amino acid sequence SEQ ID NOS: 118 to 119 and Jip-c8 corresponding to the amino acid sequence SEQ ID NOS: 122 to 123 (Fig. 1). All these proteins have the leucine zipper and directly interact with c-Jun.

[0130] Hereafter, the proteins of the present invention will be further explained.

[0131] Among the proteins of the present invention, the proteins having any one of the amino acid sequences of SEQ ID NOS: 1 to 124 are proteins for which it has been found that they interact with the c-Jun protein, i.e., form a complex, as described in the examples mentioned below. For proteins, existence of a mutant having the same function is generally expected. Further, by suitably modifying an amino acid sequence of a protein, a mutant having the same function can be obtained. Therefore,

proteins that have any one of the amino acid sequences of SEQ ID NOS: 1 to 124 including deletion, substitution or addition of one or several amino acid residues and interact with the c-Jun protein also fall within the scope of the proteins of the present invention. The term "several" used herein means preferably within 5, more preferably within 2. Further, proteins that show a homology of usually 15% or more, preferably 90% or more, more preferably 95% or more to any one of the amino acid sequences of SEQ ID NOS: 1 to 124 and interact with the c-Jun protein also fall within the scope of the protein of the present invention.

[0132] An amino acid sequence of a protein can be modified by modifying a nucleotide sequence of DNA encoding the protein using a well-known means such as site-directed mutagenesis and expressing DNA of which nucleotide sequence is modified. Among such modified proteins, those that interact with the c-Jun protein fall within the scope of the protein of the present invention. The interaction with the c-Jun protein can be measured by a known method for measuring an interaction, and examples include the method of detecting formation of a complex mentioned in the examples described later.

[0133] The proteins of the present invention may be fused with another protein and thus provided as a fusion protein.

[0134] The nucleic acids of the present invention are nucleic acids encoding the proteins of the present invention. The nucleic acids are usually RNA or DNA. Examples of the nucleic acids of the present invention include nucleic acids having any one of the nucleotide sequences of SEQ ID NOS: 126 to 254. These nucleic acids are nucleic acids of which nucleotide sequences were determined in the examples mentioned below. For a gene, existence of a gene encoding the same product, but having a different nucleotide sequence, or a gene encoding a mutant having the same function is expected. Further, by suitably modifying a nucleotide sequence, a gene encoding the same

product or a mutant having the same function can be obtained. Therefore, nucleic acids having a nucleotide sequence similar to any one of the nucleotide sequences of SEQ ID NOS: 126 to 254 and encoding a protein that interacts with the c-Jun protein also fall within the scope of the nucleic acids of the present invention. Examples of such nucleic acids having a similar nucleotide sequence include nucleic acids that hybridize with a nucleic acid having a nucleotide sequence complementary to any one of the nucleotide sequences of SEQ ID NOS: 126 to 254 under a stringent condition, and nucleic acids having a nucleotide sequence showing a homology of usually 16% or more, preferably 90% or more, more preferably 95% or more to any one of the nucleotide sequences of SEQ ID NOS: 126 to 254.

[0135] The stringent condition referred to here corresponds to, for example, that of hybridization using DIG Easy Hyb (Roch Diagnostics) at 42°C followed by washing in 0.1 x SSC/0.1% SDS for 15 minutes at 60°C. Homology of nucleotide sequences is obtained as a rate of number of nucleotides matching in alignment of the nucleotide sequences to be compared to the nucleotide number of the chain length of the nucleotide sequences. Further, homology of amino acid sequences is obtained as a rate of number of amino acid residues matching in alignment of the amino acid sequences to be compared to the amino acid number of the chain length of the amino acid sequences.

[0136] Whether a DNA encodes a protein that interacts with the c-Jun protein can be easily confirmed by expressing a protein from that DNA and confirming whether the expressed protein interacts with the c-Jun protein using the aforementioned method.

[0137] The nucleic acids of the present invention can be obtained by a conventional method on the basis of the elucidated nucleotide sequences. For example, it may be synthesized by a chemical synthesis method, or it may be obtained by RT-PCR using suitably designed primers from a mRNA prepared from cells or tissue expressing a protein

that interacts with the c-Jun protein.

[0138] <2> Use of the proteins of the present inventions and others.

The proteins and genes of the present invention can be applied as an inhibitor that blocks transcription function, gene replication function and so forth as for c-Jun in gene therapy etc. by utilizing the novel function obtained by the nucleic acid sequences (function enabling binding with c-Jun in this case). The basis for this originates in the fact that the genes of the proteins of the present invention are detected after undergoing competitive process of screening repeated multiple times. Genes detected by this method exhibit a certain number distribution, and a gene having higher competitive power will be detected in a larger number. This means that a gene of which clones are detected by this method in a larger number should have a higher competitive power, and it more effectively functions as a blocking agent or inhibitor.

[0139] As for use of the proteins of the present invention and genes encoding them, as *in vitro* applications, they can be applied in, for example, evolutionary molecular engineering using a cell-free protein synthesis system or genomic function analysis by utilizing the novel function provided by the proteins, genes or nucleic acid sequences according to the present invention. In this case, analysis utilizing cotranslation screening and selection of assigning molecules is extremely effective. This is because the cotranslation screening/selection method makes it possible to comprehensively detect proteins that directly or indirectly interact with a bait protein. Furthermore, in analysis of interactions between IVVs, IVV and C-terminal labeled protein etc., they can also be use as a "target molecule (bait protein)". Examples of general methods for analyzing an interaction include, for example, microarray method, fluorescence correlation spectroscopy

(FCS/FCCS), fluorescence imaging analysis, fluorescence resonance energy transfer method, evanescent-field molecular imaging method, fluorescence depolarization method, surface plasmon resonance method, enzyme linked immunosorbent assay and so forth. Specific examples of the cell-free protein synthesis system include wheat germ extract, rabbit reticulocyte lysate, *Escherichia coli* S30 extract and so forth. By adding a protein, gene or nucleic acid sequence as a translation template according to the present invention to any of these cell-free protein synthesis systems, simultaneously adding 1 to 100 μ M of modification agent in the case of C-terminal labeling, and maintaining the system at 25 to 37°C for 1 to several hours, a C-terminal modified protein is synthesized. In the case of assigning, only by adding a protein, gene or nucleic acid sequence as a translation template according to the present invention to the cell-free protein synthesis system and maintaining the system at 25 to 37°C for 1 to several hours, an assigning molecule is synthesized.

[0140] Further, as for in vivo applications, by utilizing the novel function provided by the proteins, genes or nucleic acid sequences according to the present invention, for example, a protein modified for separation and labeled for detection (double modified protein) synthesized in a cell-free protein synthesis system can be used as it is for a subsequent purification process, detection process, or direct introduction into cells. Specific examples of cell expression system include any kind of cells of from bacteria such as *Escherichia coli*, *Bacillus subtilis* and thermophilic bacteria, yeast to cultured cells of insects, mammals and so forth, cells of threadworm, drosophila, zebra fish, mouse and so forth. By directly introducing the aforementioned C-terminal labeled or assigned double modified protein into these cells, an objective protein can be blocked. Alternatively, it is also possible to introduce the aforementioned gene or nucleic acid of the present invention and utilize the gene

or nucleic acid as it is as an antisense sequence or RNAi sequence to block expression of an objective nucleic acid, or they can be expressed in a cell and utilized as a protein or assigning molecule to block a protein having an interacting action. When a protein is used in the C-terminal labeling method, by simultaneously introducing 1 to 100 μ M modification agent for C-terminal labeling into cells using electroporation, microinjection or the like and maintaining the cells at the optimum growth temperature of the cells for several hours, a modified protein is synthesized. In the case of assigning, by introducing a template of an assigning molecule having the aforementioned gene or nucleic acid sequence of the protein of the present invention into cells and maintaining the cells at the optimum growth temperature of the cells for several hours, an assigning molecule is synthesized. The synthesized double modified protein can be collected by disrupting the cells and used for the subsequent purification process or detection process. Further, it can be used as it is in the cells for the detection process.

[0141] Hereafter, the use of the proteins of the present invention and others will be further explained.

[0142] The detection method of the present invention is a method of utilizing the proteins of the present invention as a bait in detection of interaction between the bait and a prey.

[0143] Preferably, the method is mainly characterized in that the bait and prey are modified for separation and labeled for detection in a specific manner, and the prey is produced by translation in a cell-free translation system in the presence of the bait to contact the bait and prey. In this specification, contacting a bait and a prey by producing the prey by translation in a cell-free translation system in the presence of the bait is also referred to as "cell-free cotranslation".

[0144] In this specification, the terms of "bait" and "prey" have the meanings usually used in the technical

field of analysis of interaction between substances. That is, a protein, nucleic acid or the like as a known substance is called "bait", and a protein, nucleic acid or the like as a substance that interacts with the bait is called "prey". In the present invention, the prey is preferably a protein.

[0145] The bait used herein may be the protein of the present invention, or a complex constituted by arbitrary components including protein (including peptide), nucleic acid, or ligand such as antibody and hormone, metal and so forth, so long as it contains the protein of the present invention, and it may be a natural substance or artificial substance. The bait is not particularly limited as for the molecular weight and so forth. Examples include, for example, in the case of protein, a functional domain, a full-length protein containing a functional domain and so forth. If a prey library is used, use of full-length proteins enables comprehensive detection.

[0146] Further, as the prey, a protein is preferably used. The prey is not particularly limited as for the molecular weight and so forth.

[0147] Preferably, the detection method of the present invention is mainly characterized in that, in the detection of an interaction of the bait and prey, the bait and prey are modified for separation and labeled for detection in a specific manner, and cell-free cotranslation is performed as described above. Therefore, a preferred configuration of the detection method of the present invention may be the same as that of a usual method for detecting an interaction between a bait and a prey comprising contacting the bait and prey and detecting a complex formed by the contact, except that the bait and prey are modified for separation and labeled for detection in a specific manner, and cell-free cotranslation is performed.

[0148] Although the modification for separation and labeling for detection of the bait and prey are arbitrarily performed so that they are suitable for the detection of

the complex, they should be performed so that both of the bait and prey should not be labeled with a label for detection or modified for separation. Therefore, the prey is used as a fusion protein with a protein that can be used as a label for detection or an assigning molecule, and the bait correspondingly has a modification for separation.

[0149] When the prey is used as a fusion protein, the bait should have a modification for separation. When the bait is a protein, the bait is preferably produced in a cell-free translation system by translation of mRNA encoding a fusion protein containing the bait as a fusion protein with a protein that can be used as a modification for separation in the cell-free translation system.

[0150] Examples of the modification for separation in the case where the bait is a protein include formation of a fusion protein with the GST protein, CBP used for the TAP method etc. (this can be separated by using affinity with calmodulin beads), protein A (this can be separated by using IgG-protein A affinity) as a protein, or any of various antibody tags etc. as an affinity tag. When the bait itself has a property that it can be used as a modification for separation, the bait can be used as it is as a bait having a modification for separation. Examples of the modification for detection of the prey include formation of a fusion protein with a fluorescent protein such as GFP (green fluorescent protein).

[0151] Preparation of mRNA encoding such a fusion protein mentioned above and translation of this mRNA in a cell-free translation system can be performed by usual methods. The mRNA may be mRNA produced by transcription of DNA in a cell-free transcription and translation system.

[0152] When the prey is an assigning molecule, arbitrary modification for separation can be added to the bait. When the bait is a protein, the aforementioned examples of the modification for separation can be used. In addition, when the bait is a nucleic acid, drug or the like, examples of the modification for separation include

use of biotin etc. that interact with streptavidin or avidin. When the bait itself has a property that it can be used as modification for separation, the bait can be used it is as a bait having modification for separation.

[0153] An assigning molecule means a molecule assigning a phenotype and a genotype. The assigning molecule is usually a molecule comprising a genotype molecule containing a nucleic acid having a nucleotide sequence reflecting a genotype and a phenotype molecule containing a protein relating to expression of phenotype, which are bound to each other. By using a prey as this protein, the prey can be used as an assigning molecule. Such an assigning molecule can be formed by performing translation of mRNA encoding a prey in a cell-free translation system so that the translated prey should associate with the mRNA, or performing transcription and translation of DNA encoding a prey in a cell-free transcription and translation system so that the translated prey should associate with the DNA. Therefore, by allowing a bait to exist during the production, cell-free cotranslation can be attained. That is, the cell-free cotranslation can be performed by the following scheme (1) or (2).

[0154] (1) By performing translation of mRNA encoding the prey in the presence of the bait in a cell-free translation system so that the translated prey should associate with the mRNA, the prey is produced in the cell-free translation system, and thereby the bait and prey are brought into contact with each other.

[0155] (2) By performing transcription and translation of DNA encoding the prey in the presence of the bait in a cell-free transcription and translation system so that the translated prey should associate with the DNA, the prey is produced in the cell-free translation system, and thereby the bait and prey are brought into contact with each other.

[0156] Hereafter, the embodiments of (1) and (2) mentioned above will be explained.

[0157] In the embodiment of (1), the translated prey preferably associates with the mRNA, because the mRNA has a spacer region bound to the 3' end and a peptide acceptor region bound to the spacer region and containing a group that can bind to a peptide by transpeptidation reaction. Examples of the method for detecting an interaction using such an assigning molecule include the *in vitro* virus method.

[0158] The mRNA is preferably a nucleic acid containing a 5' untranslation region including a transcription promoter and a translation enhancer, an ORF region encoding a prey and binding to the 3' end side of the 5' untranslation region, and a 3' end region including a poly-A sequence and binding to 3' end side of the ORF region. Preferably, an expression amplification sequence containing an SNNS (S is G or C) sequence on the 5' end side of the poly-A sequence (for example, a sequence recognizable by the restriction enzyme *XhoI*) is further included. The mRNA may or may not have a Cap structure at the 5' end.

[0159] The poly-A sequence is a poly-A continuous chain of at least 2 or more residues comprising dA and/or rA as single kind of residues or mixture of two kinds, and the poly-A chain consists of, preferably 3 or more residues, more preferably 6 or more residues, still more preferably 8 or more residues.

[0160] One of the factors affecting the translation efficiency is a combination of the 5' UTR comprising a transcription promoter and a translation enhancer and the 3' end region including a poly-A sequence. The effect of the poly-A sequence of the 3' end region is usually exerted with a length of ten or less residues. As the transcription promoter of the 5' UTR, T7/T3, SP6, and so forth can be used, and no particular limitation is imposed. SP6 is preferred, and it is particularly preferable to use SP6, especially when a sequence containing an omega sequence or a part of omega sequence is used as the

translation enhancer sequence. The translation enhancer is preferably a part of the omega sequence, and as the part of the omega sequence, one containing a part of the omega sequence of TMV (O29, refer to Gallie D.R., Walbot V., Nucleic Acids Res., vol. 20, 4631-4638 (1992), and WO02/48347, Fig. 3) is preferred.

[0161] Further, for the translation efficiency, the combination of the *XhoI* sequence and a poly-A sequence is preferred in the 3' end region. Furthermore, a combination of the downstream portion of the ORF region, i.e., the upstream region of the *XhoI* sequence having an affinity tag, and a poly-A sequence is preferred. The affinity tag sequence may be any sequence for utilizing a means that can detect a protein such as an antigen-antibody reaction, and no limitation is imposed. The affinity tag is preferably the Flag-tag sequence or His-tag sequence, which is a tag for affinity separation analysis based on an antigen-antibody reaction. As for the effect of the poly-A sequence, an affinity tag such as the Flag-tag attached with the *XhoI* sequence and further attached with a poly-A sequence increases the translation efficiency. As for the His-tag, even a His-tag having a configuration not containing the *XhoI* sequence also exhibits sufficient translation efficiency, and thus is effective.

[0162] Such a configuration effective for improvement of translation efficiency is also effective for assignment efficiency.

[0163] If SP6+O29 and Flag+*XhoI*+A_n (n = 8) or His+A_n (n = 8), for example, are used as the 5' UTR and the 3' end region, respectively, the 5' UTR and the 3' end region would have lengths of about 49 bp and about 38 or 26 bp, respectively, and thus they have such a length that they can be incorporated into primers for PCR as an adaptor region. Therefore, a coding region having such a 5' UTR and 3' end region can be easily produced by PCR from any of vectors, plasmids and cDNA libraries. In the coding region, translation may occur beyond the ORF region. That is,

there may not be a stop codon at the end of the ORF region.

[0164] The peptide acceptor region is not particularly limited, so long as it can bind to the C-terminal of a peptide. For example, puromycin and 3'-N-aminoacylpuromycin aminonucleosides (PANS-amino acids) including PANS-amino acids corresponding to all amino acids such as PANS-Gly in which the amino acid portion is glycine, PANS-Val in which the amino acid portion is valine, and PANS-Ala in which the amino acid portion is alanine can be utilized. Further, 3'-N-aminoacyladenine aminonucleosides (AANS-amino acids), in which a 3'-aminoacyladenine and an amino acid are bonded via an amide bond as a chemical bond formed as a result of dehydration condensation of the amino group of the 3'-aminoacyladenine and the carboxyl group of the amino acid, corresponding to all amino acids, for example, AANS-Gly in which the amino acid portion is glycine, AANS-Val in which the amino acid portion is valine, AANS-Ala in which the amino acid portion is alanine, and so forth can also be used. Furthermore, nucleosides and nucleosides bound with an amino acid via an ester bond can also be used. In addition, any of substances formed with a bonding scheme that can chemically bond a nucleoside or a substance having a chemical structure similar to that of nucleoside and an amino acid or a substance having a chemical structure similar to amino acid can be used.

[0165] The peptide acceptor region preferably comprises puromycin or a derivative thereof, or puromycin or a derivative thereof and one or two residues of deoxyribonucleotides or ribonucleotides. The term "derivative" used in this case means a derivative that can bind to the C-terminal of peptide in a protein translation system. The puromycin derivative is not limited to those having the total puromycin structure, and includes those having the puromycin structure a part of which is eliminated. Specific examples of the puromycin derivative include PANS-amino acids, AANS-amino acids and so forth.

[0166] Although the peptide acceptor region may have a structure consisting only of puromycin, it preferably has a nucleotide sequence comprising DNA and/or RNA of one or more residues at the 5' end side. As such a sequence, dC-puromycin, rC-puromycin, and so forth, more preferably, a CCA sequence comprising dCdC-puromycin, rCrC-puromycin, rCdC-puromycin, dCrC-puromycin or the like and imitating the 3' end of aminoacyl-tRNA (Philipps, G.R., Nature 223, 374-377 (1969)), is suitable. As for the type of nucleotide, preference is higher in the order of C > (U or T) > G > A.

[0167] The spacer region is preferably a PEG region containing polyethylene glycol as a main component. The spacer region usually contains, in addition to the PEG region, a donor region that can bind to the 3' end of a nucleic acid.

[0168] The donor region that can bind to the 3' end of nucleic acid usually consists of one or more nucleotides. The number of nucleotides is usually 1 to 15, preferably 1 to 2. The nucleotides may be a ribonucleotide or a deoxyribonucleotide. The donor region may have a modification substance.

[0169] The sequence of the 5' end of the donor region affects the ligation efficiency with the coding region encoding the prey. In order to attain ligation of the coding region and the spacer region, it is required to include at least one or more residues, and at least one residue of dC (deoxycytidylic acid) or two residues of dCdC (dideoxycytidylic acid) is preferred for an acceptor having a poly-A sequence. As for the type of nucleotide, preference is higher in the order of C > (U or T) > G > A.

[0170] The PEG region contains polyethylene glycol as a main component. The expression "contains polyethylene glycol as a main component" used herein means that the total number of nucleotides contained in the PEG region is 20 or less, or the average molecular weight of the polyethylene glycol is 400 or more. It preferably means

that the total number of nucleotides is 10 or less, or the average molecular weight of the polyethylene glycol is 1000 or more.

[0171] The average molecular weight of the polyethylene glycol in the PEG region is usually 400 to 30,000, preferably 1,000 to 10,000, more preferably 2,000 to 8,000. If the molecular weight of the polyethylene glycol is lower than about 400, a posttreatment for assignment translation may be required for assignment translation of a genotype molecule containing the spacer region (Liu, R., Barrick, E., Szostak, J.W., Roberts, R.W., *Methods in Enzymology*, vol. 318, 268-293 (2000)). However, if PEG having a molecular weight of 1000 or more, preferably 2000 or more, is used, highly efficient assignment can be attained only by assignment translation, and therefore the posttreatment for the translation becomes unnecessary. Further, when the molecular weight of the polyethylene glycol increases, stability of the genotype molecule tends to increase, and in particular, the stability becomes favorable with a molecular weight of 1000 or more. If the molecular weight is 400 or less, properties thereof are not different so much from those of a DNA spacer, and it may become unstable.

[0172] By having a spacer region containing polyethylene glycol as a main component, it becomes possible to form an assigning molecule not only in a cell-free translation system of rabbit reticulocytes, but also in a cell-free translation system of wheat germ, the stability of the genotype molecule in both translation systems is markedly improved, and it becomes unnecessary to perform any treatment after the translation.

[0173] In the embodiment of (2), it is preferred that DNA encodes a fusion protein of a protein and streptavidin or avidin, DNA is labeled with biotin, and a translated prey associates with the DNA because transcription and translation is carried out in a state that one DNA molecule is contained in one compartment of emulsion. Examples of

the method for detecting an interaction using such an assigning molecule include the STABLE method.

[0174] The emulsion is usually a W/O type emulsion formed by mixing two kinds of surface active agents, mineral oil and a reaction mixture of cell-free transcription and translation system. In order to form a W/O type emulsion, it is usually necessary for the surface active agents to have an HLB (hydrophile-lipophile balance) value of 3.5 to 6. The HLB value of mixed two kinds of surface active agents is calculated from the HLB values of the individual surface active agents by using a simple equation. For example, if Span 85 (HLB = 1.8) and Tween 80 (HLB = 15.0) are mixed in volumes of 40.2 μ l and 9.8 μ l, respectively, the mixture has an HLB value of 4.4. The ratio of the surface active agents and mineral oil is usually 1:18 (volume ratio). Further, the ratio of the reaction mixture is 1 to 50% (volume ratio) with respect to the whole emulsion, and it is usually 5%. The emulsion can be formed by adding the reaction mixture as several divided portions to a mixture of the surface active agents and mineral oil at a low temperature with stirring and mixing them. The reactions of transcription and translation can be started by raising the temperature of the emulsion.

[0175] The preparation of DNA encoding a prey, and transcription and translation of such a DNA in a cell-free transcription and translation system can be performed in a usual manner.

[0176] As described above, by labeling the bait and prey for detection and modifying them for separation in particular schemes, a complex formed by cell-free cotranslation can be specifically detected.

[0177] As for the cell-free cotranslation of a bait and a prey, the cell-free translation system (including cell-free transcription and translation system) in which the cell-free cotranslation is performed may be any of systems of *E. coli*, rabbit reticulocytes, wheat germs and so forth. Although formation of assigning molecules is

quite unstable with *E. coli* in the *in vitro* virus method, it has been confirmed that it is stable in a system of rabbit reticulocytes (Nemoto N., Miyamoto-Sato E., and Yanagawa H., FEBS Lett. 414, 405 (1997); Roberts R.W., Szostak J.W., Proc. Natl. Acad. Sci. USA, 94, 12297 (1997)), and it has been further confirmed that it is still more stable in a system of wheat germ (Japanese Patent Laid-open No. 2002-176987). For the STABLE method, the system may be any of systems of *E. coli*, rabbit reticulocyte, wheat germ and so forth.

[0178] The conditions for the translation and transcription in the cell-free cotranslation are suitably selected depending on a cell-free translation system to be used.

[0179] The templates of the bait and prey added to the cell-free translation system may be either RNA or DNA, so long as the cell-free translation system is a cell-free transcription and translation also causing transcription.

[0180] Hereafter, an example of a translation template preferred for use as a bait will be explained.

[0181] As a bait used in the cotranslation screening of this embodiment, used is a translation template characterized by comprising a coding portion having information for translation into a protein and a PEG spacer portion as shown in Fig. 7. The coding portion has information for translation into a protein, and it may be any sequence. However, it is preferably characterized by having an acceptor (A sequence) in a 3' end region of the coding portion, or having an acceptor (A sequence) in a 3' end region of the coding portion and a translation amplification sequence (X sequence) 5'-upstream from the A sequence. It contains a short poly-A sequence as the A sequence of the coding portion. The short poly-A sequence is usually a sequence comprising 2 to 10 nucleotides of A. It is characterized by having a sequence having (C or G)NN(C or G) sequence, for example, a *XhoI* sequence, as the X sequence. The PEG spacer portion has a PEG region

containing polyethylene glycol as a main component, a donor region for ligation with the coding portion, and a CCA region at the 3' end. Although the PEG spacer portion may consist only of the donor region or CCA region, it is preferably has a configuration comprising the PEG region containing polyethylene glycol as a main component. The CCA region is characterized by not having a function of binding by a transpeptidation reaction to a protein translated from the translation template. The PEG region is characterized by having a molecular weight of the polyethylene glycol of 500 or more. Further, it is characterized by containing at least one function-imparting unit (F) in the donor region and/or the CCA region. It is characterized in that the function-imparting unit (F1 and/or F2) immobilizes or labels with fluorescence the translation template and/or a protein translated from the translation template. As the immobilization substance, biotin and so forth are contemplated, and as the fluorescent substance, fluorescein, Cy5, rhodamine green (RhG) and so forth can be contemplated. The present invention relates to these coding portion, translation template, and libraries thereof, as well as a protein translated on a ribosome and library thereof.

[0182] The translation template of a bait (Fig. 7, A) comprises a coding portion derived from a coding molecule (Fig. 7, B) and a PEG spacer portion derived from a PEG spacer molecule (Fig. 7, C). In this embodiment, a PEG spacer portion can be ligated to the coding portion to improve stability thereof, and thus translation efficiency can be improved, basically regardless of the sequence of the coding portion. However, it is further possible to further improve the translation efficiency depending on the configuration of the coding portion or the type of the PEG spacer portion. The details thereof are described below.

[0183] The coding portion of this embodiment (Fig. 7, B) comprises a 5' end region, an ORF region, and a 3' end region, and it may or may not have a Cap structure at the

5' end. Further, the sequence of the coding portion is not particularly limited, and use thereof as one incorporated into any vector or plasmid can be contemplated. The 3' end region of the coding portion includes one having a poly-A x 8 sequence as the A sequence or one having, as the X sequence, the XhoI sequence or a sequence of SNNS (S is G or C) as a sequence of 4 or more nucleotides, and XA as a combination of the A sequence and X sequence. A configuration that a Flag-tag sequence is included as an affinity tag sequence upstream from the A sequence, X sequence, or XA sequence is contemplated. The affinity tag sequence used here may be a sequence for using any means that enables detection or purification of a protein, for example, those utilizing an antigen-antibody reaction such as HA-tag and protein A of IgG (z domain), His-tag, and so forth. As for factors affecting the translation efficiency, the combination of the XA sequence is important. The first four nucleotides of the X sequence are important, and one having a sequence of SNNS is preferred. Further, the 5' end region comprises a transcription promoter and a translation enhancer. As the transcription promoter, T7/T3, SP6, and so forth can be used, and no particular limitation is imposed. However, for a cell-free translation system of wheat, a sequence containing an omega sequence or a part of omega sequence is preferably used as the translation enhancer sequence; and SP6 is preferably used as the promoter. The part of the omega sequence is one containing a part of the omega sequence of TMV (O29, refer to Gallie D.R., Walbot V., *Nucleic Acids Res.*, vol. 20, 4631-4638 (1992) and WO02/48347, Fig. 3). The ORF region of the coding portion may be any sequence comprising DNA and/or RNA. It may be a gene sequence, exon sequence, intron sequence, random sequence, or any natural sequence or artificial sequence, and the sequence is not limited.

[0184] The PEG spacer molecule of this embodiment (Fig. 8, C) comprises a CCA region, a PEG region, and a donor region. The minimum essential component is the donor

region. As for the factors affecting the translation efficiency, one having not only the donor region but also the PEG region is preferred, and it preferably has puromycin which does not have an ability to bind with an amino acid. The molecular weight of the polyethylene glycol in the PEG region is 400 to 30,000, preferably 1,000 to 10,000, more preferably 2,000 to 6,000. Further, the CCA region may have a configuration including puromycin or a configuration not including puromycin. As puromycin, puromycin and 3'-N-aminoacylpuromycin aminonucleosides (PANS-amino acids) including PANS-amino acids corresponding to all amino acids such as PANS-Gly in which the amino acid portion is glycine, PANS-Val in which the amino acid portion is valine, and PANS-Ala in which the amino acid portion is alanine can be utilized. Further, 3'-N-aminoacyladenine aminonucleosides (AANS-amino acids), in which a 3'-aminoacyladenine and an amino acid is bonded via an amide bond as a chemical bond formed as a result of dehydration condensation of the amino group of the 3'-aminoacyladenine and the carboxyl group of the amino acid, corresponding to all amino acids, for example, AANS-Gly in which the amino acid portion is glycine, AANS-Val in which the amino acid portion is valine, AANS-Ala in which the amino acid portion is alanine, and so forth can also be used. Furthermore, nucleosides and nucleosides bound with an amino acid via an ester bond can also be used. In addition, any of substances formed with a bonding scheme that can chemically bond a nucleoside or a substance having a chemical structure similar to that of nucleoside and an amino acid or a substance having a chemical structure similar to amino acid can be used. For this translation template, any substances corresponding to the aforementioned puromycin derivatives of which amino group lacks the ability to bind to an amino acid, and a CCA region lacking puromycin are also contemplated. However, by incorporating puromycin that cannot bind with a protein on a ribosome, the translation efficiency can be further

enhanced. Although the reason for this is not certain, it may possible that puromycin that cannot bind with a protein stimulates a ribosome to enhance the turnover. A nucleotide sequence comprising DNA and/or RNA of one or more residues is preferably contained on the 5' end side of the CCA region (CCA). As for the type of nucleotide, preference is higher in the order of C > (U or T) > G > A. As such a sequence, dC-puromycin, rC-puromycin, and so forth, more preferably, a CCA sequence comprising dCdC-puromycin, rCrC-puromycin, rCdC-puromycin, dCrC-puromycin or the like and imitating the 3' end of aminoacyl-tRNA (Philipps, G.R., Nature 223, 374-377 (1969)) is suitable. In one embodiment of the present invention, these puromycins are made incapable of binding with an amino acid in a certain manner.

[0185] The PEG spacer portion of this embodiment may have a configuration containing a modification substance (F1 and/or F2). With this characteristic, it can be used as a tag for collection, reuse by purification, or immobilization of translation template. Those comprising at least one residue of nucleotide of DNA and/or RNA incorporated with any of various separation tags such as fluorescent substance, biotin, and His-tag may be possible. Further, if SP6+O29 and Flag+XhoI+An (n = 8) are used as the 5' end region and the 3' end region of the coding portion, respectively, for example, the lengths of the 5' end region and the 3' end region are about 60 bp and about 40 bp, respectively, and thus they have such a length that they can be designed in primers for PCR as an adaptor region. This provides a novel advantage. That is, it becomes possible to easily prepare a coding portion having a 5' end region and 3' end region according to this embodiment by PCR from any vector, plasmid, and cDNA library, and by ligating the PEG spacer portion, instead of a 3' UTR, to this coding portion, a translation template showing a high translation efficiency can be obtained.

[0186] The ligation of the PEG spacer molecule and the

coding molecule according to this embodiment may be attained any method such as usual methods utilizing a DNA ligase or those based on a photoreaction, and the method is not particularly limited. In the ligation using an RNA ligase, as factors in the coding portion affecting the ligation efficiency, the A sequence of the 3' end region is important. It is a poly-A continuous chain consisting of at least two, preferably 3 or more, more preferably 6 to 8, of single kind or mixed kinds of residues selected from dA and/or rA. The DNA and/or RNA sequence of the 5' end of the donor region of the PEG spacer portion affects the ligation efficiency. In order to ligate the coding portion and PEG spacer portion with an RNA ligase, it is required to contain at least one or more residues, and for an acceptor having a poly-A sequence, at least 1 residue of dC (deoxycytidylic acid) or two residues of dCdC (dideoxycytidylic acid) is preferred. As for the type of nucleotide, preference is higher in the order of C > (U or T) > G > A. Furthermore, it is preferable to add polyethylene glycol of the same molecular weight as the PEG region during the ligation reaction.

[0187] Hereafter, an example of a translation template preferably used as a prey will be explained.

[0188] As a prey in cotranslation screening according to this embodiment, a protein of which C-terminal is modified with a translation template as represented in Fig. 9 (i.e., assigning molecule) is used. The translation template comprises a coding portion having information for translation into a protein and a PEG spacer portion. The coding portion has an A sequence at the 3' end, and the A sequence comprises a short poly-A sequence. The PEG spacer portion is characterized in that polyethylene glycol in the PEG region containing polyethylene glycol as a main component has a molecular weight of 400 or more, and the donor region and/or the CCA region contains at least one modification substance (F1 and/or F2). Further, the CCA region is characterized by having a function of binding by

transpeptidation to a protein translated from the translation template, and the CCA region typically has puromycin. Further, it is characterized in that the modification substance (F1 and/or F2) immobilizes or labels with fluorescence the translation template and/or a protein translated from the translation template. As the immobilization substance, biotin and so forth are contemplated, and as the fluorescent substance, fluorescein, Cy5, rhodamine green (RhG) and so forth can be contemplated. The present invention relates to these coding portion, translation template, and libraries thereof, as well as a protein synthesized by translation on a ribosome (i.e., assigning molecule) and a library of such proteins (i.e., assigning molecules).

[0189] The prey is a protein synthesized by translation utilizing the translation template, of which C-terminal is modified with the translation template (Fig. 8, A, assigning molecule), and has characteristics in the translation template (Fig. 8, B) and the configuration of a protein of which C-terminal is modified with PEG (Fig. 8, C). It will be described in detail below.

[0190] The PEG spacer portion of the translation template (Fig. 8, B) is the same as that of the aforementioned translation template preferred for use as an bait except that it is characterized in that puromycin can bind with an amino acid. Further, the coding portion is also the same as that of the aforementioned translation template preferred for use as a bait. However, as for a configuration suitable for assignment, in particular, it is important to use an A sequence as the 3' end region, and this markedly increase the assignment efficiency of the total proteins and markedly decrease the amount of free proteins. Also in this case, if SP6+O29 and Flag+XhoI+An (n = 8) are used as the 5' end region and the 3' end region of the coding portion, respectively, for example, the lengths of the 5' end region and the 3' end region are about 60 bp and about 40 bp, respectively, and thus they

have such a length that they can be designed in primers for PCR as an adaptor region. This makes it possible to easily prepare a coding portion having a 5' end region and 3' end region according to this embodiment by PCR from any vector, plasmid, and cDNA library, and by ligating the PEG spacer portion, a translation template showing a high assignment efficiency can be obtained.

[0191] When the coding portion of the protein of which C-terminal is modified with PEG according to this embodiment (Fig. 8, C) is not used in detection of an interaction of proteins, i.e., when the protein is use for, for example, FCCS measurement, fluorescence reader, protein chip, and so forth, it may be intentionally cleaved with an RNase A or the like. By the cleavage, difficulty of detection of an interaction between proteins due to inhibition by the coding portion can be eliminated. Further, it is also possible to immobilize such a simple assigning molecule on a plate, bead, or slide glass.

[0192] The cell-free cotranslation will be explained with reference to Fig. 9. As shown in Fig. 9, a prey is translated *in vitro* in the presence of a bait. As shown in Fig. 9, A and B, there are a case where the bait is a protein, and it is translated simultaneously with the prey in a cell-free translation system, and a case where the bait is a nucleic acid, hormone or the like, and it is added to a cell-free translation system. As shown in Fig. 9, the prey is made into a fusion protein or assigning molecule.

[0193] The complex may be formed by binding of a bait and one prey (I), or by binding of another prey to a prey binding to a bait (II).

[0194] Because the detection method of the present invention enables *in vitro* formation of the complex, interactions between proteins, nucleic acid and protein, and so forth can be consistently detected *in vitro*.

[0195] When the bait is a protein, examples of the bait include a protein consisting only of a functional

domain for an interaction with an objective protein, a protein including a functional domain, a protein of full length, and so forth. If a protein of full length is used, it is expected that it has multiple functional domains, and therefore it favorably becomes possible to more comprehensively detect preys. The protein of full length may be a single protein having a full length, or an assembly of two or more baits from which a protein of full length can be reconstructed.

[0196] The bait may be a complex as shown in Fig. 10, and this is called "complexed bait". By using such a complex, nonspecific adsorption can be further reduced, and it becomes possible to more comprehensively detect preys as the same effect as that of the full length protein.

[0197] As described above, as a complex contemplated for the cell-free cotranslation, a complex of a single bait and a single prey, a complex of a complexed bait and a prey, a complex of a bait and multiple preys, and a complex of a complexed bait and multiple preys are possible. Therefore, an interaction detectable by the detection method of the present invention includes not only a direct interaction between a bait and a prey, but also an indirect interaction for forming a complex.

[0198] It is considered that the most important factor in the cell-free cotranslation according to the present invention is that a protein is folded in a native state and in an undenatured state immediately after translation, a bait and a prey, a bait and another bait, or a prey and another prey, which should interact with each other, should coexist in a cell-free translation system, and thus they can promptly interact with each other. This is supported by the fact that a more superior result could be obtained by cotranslation compared with separate translation followed by coexistence by mixing. That is, it is considered that this is because a protein translated *in vitro* in a native folding state can encounter a protein, nucleic acid or the like, and therefore prompt formation of

a complex by an interaction becomes possible.

[0199] The conventional methods of detecting an interaction require expression in *E. coli* and purification of a bait in a large amount. For example, when an interaction of a bait and a prey is expressed in a cell by the TAP method or the like, at least one month of preparation is needed. Further, the mRNA displaying method employing the pull-down method based on a GST fusion protein has problems that it takes at least 2 or 3 weeks because of the large amount expression in *E. coli* and purification of the bait, a substance that cannot be expressed in *E. coli* cannot be used as the bait, and so forth, and it further requires addition of bait in an amount 50 to 100 times the amount of prey to cause interaction with the prey. In the cell-free cotranslation, it becomes that only addition of almost equal weight of mRNA or DNA template to a cell-free translation system is sufficient, and it becomes completely unnecessary to express the bait in cells. Thus, the operation time can be markedly shortened. Furthermore, with a complexed bait or full length protein, an interaction of a bait and a prey can be further enhanced and made specific, and thus detection of nonspecific bonds can be avoided. Further, by using a complexed bait, a larger number of preys that interact with the second bait thereof can be comprehensively analyzed.

[0200] Although no system realizing complex formation by an interaction and screening consistently *in vitro* has existed so far, by performing translation and screening including those for a bait completely *in vitro* according to the detection method of the present invention described above, a system that can comprehensively detect interactions between proteins or between a protein and a nucleic acid with avoiding nonspecific detection can be constructed. Therefore, the present invention also provides a screening method utilizing the detection method of the present invention.

[0201] The screening method of the present invention is characterized by forming complexes by interactions of a bait and preys during cell-free cotranslation, and analyzing a prey that interacts with the bait by screening of the complexes. Therefore, the screening method of the present invention may be the same as an ordinary screening method for a prey that interacts with a bait comprising the detection step of detecting an interaction between a bait and prey and the selection step of selecting a prey for which an interaction is detected, except that the method comprises the detection step of detecting an interaction between a bait and a prey by the detection method of the present invention.

[0202] The screening method of the present invention further comprises the preparation step of preparing a prey selected in the selection step, and it is preferable to repeat the detection step, selection step and preparation step by using the prepared prey instead of or together with the bait used in the detection step. In this embodiment, the method is constituted by, for example, 1) the step of cell-free cotranslation in a cell-free translation system in which a prey and a bait cause an interaction, 2) the step of screening for detecting a prey interacting with the bait, 3) the step of examining and analyzing the prey, and 4) the step of repeating the steps from 1) by using the prey examined and analyzed in 3), as shown in Fig. 10. The steps of 1) and 2) correspond to the detection step and selection step, and the step of 3) corresponds to the preparation step. That is, the step of contacting a prey to the bait in the detection step corresponds to the step of the cell-free cotranslation, and the steps of detecting and selecting a complex in the detection step correspond to the step of screening.

[0203] In the screening method of the present invention, the prey selected in the selection step may be used again in the detection step.

[0204] In the screening method of the present

invention, the cell-free cotranslation may be performed with a bait and a prey library, which is a group of multiple preys, so that two or more preys may be detected in the step of screening.

[0205] As shown in Fig. 9, a complexed bait and a prey may coexist, and a complex of the complexed bait and the prey may be formed by an interaction. By using a prey library in this cell-free cotranslation so that multiple kinds of preys of the prey library should coexist with the bait, and multiple complexes of the preys with the bait should be formed by interactions, multiple kinds of preys that interacts with the bait can be simultaneously and comprehensively detected in the screening. Further, if a full length protein is used as the bait, it becomes possible to comprehensively detect a larger number of preys, because a full length protein generally contains multiple functional domains for interactions.

[0206] Furthermore, as shown in Fig. 10, by forming multiple complexes of preys that interact with a complexed bait, multiple preys that interact with the complexed bait can be detected, and the second bait serves as a reinforcer of the interaction of a bait and a prey to realize a more specific interaction, which makes it possible to avoid nonspecific detection in the comprehensive detection. In evolutionary molecular engineering techniques such as the vitro virus method and the STABLE method, the prey is an assigning molecule (fusion). In the formation of complexes using a prey library or multiple kinds of preys, the preys may or may not directly interact with the bait.

[0207] When the complex obtained by the screening of complexes is an assigning molecule, a prey forming the complex may be detected by RT-PCR or PCR, and the screening may be performed again by using the PCR product as a prey (reconstruction of prey) or by using a prey analyzed from the PCR product as a new succeeding bait, as shown in Fig. 11. The method of performing the screening again by using the PCR product or performing screening by using a prey

analyzed from the PCR product as a new succeeding bait can be performed only in the evolutionary molecular engineering techniques such as the *in vitro* virus method and the STABLE method, and cannot be carried out in a method of directly analyzing a protein such as the pull-down method and the TAP method.

[0208] When an assigning molecule is used, gene sequence of a proteinic prey can be known by RT-PCR or PCR after the screening. As shown in Figs. 9 and 10, the proteinic prey referred to above is a prey interacting with a bait, a prey interacting that prey or the like, and all multiple kinds of preys interacting a bait can be comprehensively analyzed. When rescreening of a prey is further necessary, a DNA template, which is a product of RT-PCR or PCR, is transcribed, and the same cycles are repeated. Further, when a prey is determined by RT-PCR or PCR and the following sequence, it becomes possible to use that proteinic prey as a bait. If two or more kinds of preys that interact with the first bait are found, it becomes possible to form a complexed bait, and thus it becomes possible to detect a further larger number of preys.

[0209] If the cell-free cotranslation is used, it becomes possible to detect an interaction between proteins consistently *in vitro* even in the pull-down method or the TAP method. However, the assigning molecule is not formed in the TAP method, and therefore proteins must be directly analyzed in the analysis of preys. Then, if the pull-down method or the TAP method is used as the screening method in the *in vitro* virus method or the STABLE method, an assigning molecule is formed, and therefore gene sequence of a prey that causes interaction can be easily detected by RT-PCR or PCR in the analysis of the prey. Furthermore, if the cell-free cotranslation is used, it becomes possible to detect interactions between proteins consistently *in vitro* in the *in vitro* virus method or the STABLE method. Further, the number of preys is extremely large, the range of candidate preys can be narrowed down by rescreening

performed by repeating the cycles. Further, the analyzed prey can be used as a bait in the next analysis, and if the number of preys increases, complexing of the bait advances, which results in detection of further preys. As described above, use of a prey as a bait in the subsequent cycle can be easily realized only in the *in vitro* virus method, STABLE method and so forth, which use an assigning molecule. However, the mRNA display method and the like requires synthesis in *E. coli* and purification of a large amount of GST fusion protein as a new bait, and thus preparation of the bait takes time, which makes the method difficult. If the cell-free cotranslation is used, such procedures are unnecessary, and the cycles can be easily repeated.

[0210] In the screening of complexes after the cell-free cotranslation, it is preferred that preys can be screened comprehensively without breaking the complexes produced by the cell-free cotranslation. For this purpose, a device for immobilization may be imparted to the bait with an affinity tag or the like so as to detect a prey that interacts with the bait. Any kind of such a device for immobilization may be used. Examples include, for example, a method of performing two-stage screening using IgG-protein A affinity or calmodulin beads as in the conventional TAP method, and a method of performing one- or two-stage screening using streptavidin or avidin/biotin affinity, GST-tag, Flag-tag, T7-tag, His-tag or the like as in the pull-down method.

[0211] Examples of the prey library include a cDNA library (random priming library, dT priming library), random library, peptide library, hormone library, antibody library, ligand library, pharmaceutical compound library, and so forth, and any kind of library may be used. For example, if a random priming cDNA library is used as the prey library, although a full length prey cannot be expected for this library, a prey containing a functional domain can be expected. If such a library is used especially for screening using combinations with a

complexed bait or full length protein, it becomes effective for comprehensive detection of preys.

[0212] Examples of the random priming library include cDNAs obtained by random priming and incorporated into multi-cloning sites (MCS) of vectors having a 5' untranslation region (UTR) containing the promoter of the RNA polymerase of SP6 (SP6) as a transcription promoter and a part of TMV omega sequence (O29) of the tobacco mosaic virus as a translation enhancer on the 5' end side of MCS, and containing a sequence for the Flag-tag, which is a tag for affinity separation analysis based on an antigen-antibody reaction, as an affinity tag sequence on the 3' end side of MCS, so that the Flag-tag should be added to the C-terminal of a protein expressed from an insert sequence incorporated into MCS.

[0213] The aforementioned detection method of the present invention includes the step of contacting a bait and a prey to form a complex. Therefore, a method of forming a complex of a bait and a prey that interacts with the bait is provided according to this step.

[0214] The formation method of the present invention is characterized by using the protein of the present invention as a bait in the formation of a complex of a bait and a prey, which is a protein that interacts with the bait, and preferably further labeling the bait and prey for detection and modifying them for separation in particular schemes, to perform cell-free cotranslation. Therefore, a preferred configuration of the formation method of the present invention may be the same as that of an ordinary method of forming a complex of a bait and a prey comprising contacting a bait and a prey that interacts with the bait, except that the bait and prey are labeled for detection and modified for separation in particular schemes, and the cell-free cotranslation is performed. The labeling for detection and modification for separation of the bait and prey in particular schemes as well as the cell-free cotranslation may be the same as those explained for the

detection method of the present invention.

[0215] In the formation method of the present invention, not only a complex of a bait and a prey, for which interaction is known, but also a complex comprising elements for which interaction is unknown can be formed by performing the step of contacting the bait with a prey that interacts with the bait by contacting a bait with a prey library consisting of multiple kinds of preys.

[0216] Other methods for utilizing the protein of the present invention include the followings:

[0217] a method for analyzing an interaction between a protein and a substance, which is performed by fluorescence correlation spectroscopy, fluorescent imaging analysis method, fluorescence resonance energy transfer method, evanescent field molecular imaging method, fluorescence depolarization method, surface plasmon resonance method or enzyme linked immunosorbent assay using the protein of the present invention,

[0218] a method for detecting an interaction between a protein and a substance, which uses the protein of the present invention and detects the interaction by amplification of a nucleotide sequence of a coding portion bound to the C-terminal of the protein of the present invention,

[0219] a method for detecting an interaction between a protein and a substance, which uses the protein of the present invention and uses the cell-free cotranslation method or the cell-free cotranslation screening method,

[0220] a method for detecting an interaction between a protein and a substance, which uses the protein of the present invention and labels the protein with fluorescence and/or immobilizes the protein,

[0221] a method for analyzing an interaction of a protein or substance *in vitro* by using the protein of the present invention,

[0222] a method for analyzing an interaction of a protein or substance, which uses the protein of the present

invention, and utilizes the cotranslation method *in vitro*,
[0223] a method for analyzing an interaction of a protein or substance *in vivo* by using the protein of the present invention, and

[0224] the aforementioned methods for analyzing an interaction, which uses a nucleic acid encoding the protein of the present invention.

[0225] Further, the followings are also mentioned:

[0226] a method for analyzing an interaction between a protein and a target molecule, which uses a C-terminal modified protein comprising the protein and a modification agent binding to the C-terminal of the protein. The analysis of the interaction can be carried out by fluorescence correlation spectroscopy, fluorescent imaging analysis method, fluorescence resonance energy transfer method, evanescent field molecular imaging method, fluorescence depolarization method, surface plasmon resonance method or enzyme linked immunosorbent assay. The C-terminal modified protein may be immobilized. The C-terminal modified protein may be added on an array on which the target molecule is immobilized, and then the C-terminal modified protein specifically binding to the target molecule may be detected.

[0227] In the analysis method of this embodiment, the interaction is usually analyzed by contacting the modified protein of the present invention obtained above and the target molecule in a suitable combination selected depending on the type of the modification substance or reaction system and measuring change of a signal generated by the interaction between the modified protein and the target molecule among signals generated by the modified protein or the target molecule. The analysis of the interaction is carried out by, for example, fluorescence correlation spectroscopy, fluorescent imaging analysis method, fluorescence resonance energy transfer method, evanescent field molecular imaging method, fluorescence depolarization method, surface plasmon resonance method or

enzyme linked immunosorbent assay. The details of these methods are explained below.

[0228] The "target molecule" means a molecule that interacts with the modified protein of the present invention, and it may be specifically a protein, nucleic acid, sugar chain, low molecular weight compound or the like, preferably a protein or DNA.

[0229] The protein is not particularly limited so long as it has an ability to interact with the modified protein of the present invention, and it may be a protein of full length or a partial peptide containing an active site for binding. Further, it may be a protein of which amino acid sequence or function is known or unknown. It may be a synthesized peptide chain, a protein purified from an organism, a protein obtained by translation from a cDNA library using a suitable translation system and purification, or the like, and they can be used as the target molecule. The synthesized peptide chain may be a glycoprotein consisting of a synthesized peptide chain attached with a sugar chain. Among these, a purified protein of which amino acid sequence is known or a protein obtained by translation from a cDNA library and purification using suitable methods can be preferably used.

[0230] The nucleic acid is not particularly limited so long as it has an ability to interact with the modified protein of the present invention, and either DNA or RNA may be used. Further, it may be a nucleic acid of which nucleotide sequence or function is known or unknown. Preferably, a nucleic acid of which function as a nucleic acid having an ability to bind to a protein or nucleotide sequence is known or a nucleic acid obtained by cleavage with a restriction enzyme or the like and isolation from a genomic library or the like can be used.

[0231] The sugar chain is not particularly limited so long as it has an ability to interact with the modified protein of the present invention, and it may be a sugar chain of which saccharide sequence or function is known or

unknown. Preferably, an already isolated and analyzed sugar chain of which saccharide sequence or function is known is used.

[0232] The low molecular weight compound is not particularly limited so long as it has an ability to interact with the modified protein of the present invention. A compound of which function is unknown or a compound of which ability to bind to a protein is already known may also be used.

[0233] The "interaction" caused by these targets molecules with the modified protein of the present invention usually means an action caused by an intermolecular force generated by at least one of covalent bond, hydrophobic bond, hydrogen bond, van der Waals binding and binding caused by electrostatic force between a protein and a target molecule. However, this term should be construed in its broadest sense, and it should not be construed in any limitative way. The covalent bond includes a coordinate bond and dipole bond. The binding caused by electrostatic force includes, besides electrostatic bond, electric repulsion. Further, a bonding reaction, synthetic reaction and decomposition reaction caused as a result of the aforementioned action are also included in the interaction.

[0234] Specific examples of the interaction include association and dissociation of an antigen and an antibody, association and dissociation of a protein receptor and a ligand, association and dissociation of an adhesion molecule and a partner molecule, association and dissociation of an enzyme and a substrate, association and dissociation of a nucleic acid and a protein binding to it, association and dissociation of proteins in an information transmission system, association and dissociation of a glycoprotein and a protein and association and dissociation of a sugar chain and a protein.

[0235] The target molecule to be used may be modified with a modification substance and used depending on

embodiments. The modification substance is usually selected from nonradioactive modification substances such as fluorescent substances. The fluorescent substances may be any of various fluorescent dyes of, for example, fluorescein type, rhodamine type, Cy3, Cy5, eosine type, NBD type and so forth, which have a free functional group (e.g., carboxyl group, hydroxyl group, amino group etc.) and can bind to the aforementioned target substance such as proteins and nucleic acids. In addition, other compounds such as dyes may be used, and type and size of the compounds are not critical so long as they enable the modification.

[0236] Among these modification substances, a substance suitable for the method of measurement or analysis of change of signal generated due to an interaction between the target molecule and the modified protein of the present invention is used.

[0237] The aforementioned modification substance can be bound to the target molecule by a suitable method known per se. Specifically, when the target molecule is a protein, the method of modifying the C-terminal described in WO02/48347 or the like may be used. Further, when the target molecule is a nucleic acid, it can be easily modified by a method of performing PCR using an oligo DNA primer bound with a modification substance beforehand via a covalent bond or the like.

[0238] Further, the modified protein of the present invention or the target molecule used for present invention may be bound to a solid phase (i.e., immobilized) depending on the embodiment. As the method for binding to a solid phase, there are a method of binding it via the modification substance and a method of binding it via another portion.

[0239] The modification substance used in binding via the modification substance is usually a molecule specifically binding to a particular polypeptide (henceforth also referred to as a "ligand"), and a

particular polypeptide binding to the ligand (henceforth also referred to as an "adaptor protein") is bound to the solid phase. The adaptor protein also includes binding proteins, receptor proteins constituting receptors, antibodies and so forth.

[0240] Examples of combinations of the adaptor protein and the ligand include any of various receptor proteins and a ligand thereof, for example, a biotin or iminobiotin binding protein such as avidin and streptavidin and biotin or iminobiotin, maltose binding protein and maltose, G protein and guanine nucleotide, polyhistidine peptide and metal ion such as nickel or cobalt ion, glutathione-S-transferase and glutathione, DNA binding protein and DNA, antibody and antigen molecule (epitope), calmodulin and calmodulin binding peptide, ATP binding protein and ATP, estradiol receptor protein and estradiol and so forth.

[0241] Among these, preferred combinations of the adaptor protein and the ligand are biotin or iminobiotin binding protein such as avidin and streptavidin and biotin or iminobiotin, maltose binding protein and maltose, polyhistidine peptide and metal ion such as nickel or cobalt ion, glutathione-S-transferase and glutathione, antibody and antigen molecule (epitope) and so forth, and a combination of streptavidin and biotin or iminobiotin is the most preferred. These binding proteins per se are known, and DNAs coding these proteins have already been cloned.

[0242] The adaptor protein can be bound to a solid phase surface by using a method known per se. Specifically, for example, there can be used a method of utilizing tannic acid, formalin, glutaraldehyde, pyruvic aldehyde, bis-diazotized benzidine, toluene-2,4-diisocyanate, amino group, carboxyl group that can be converted into an active ester group, hydroxyl group or amino group that can be converted into phosphoramidite group, or the like.

[0243] When the binding is attained via a portion other than the modification substance, there can be used a

known method usually used for binding a protein, nucleic acid, sugar chain or low molecular weight compound to a solid phase. Specifically, there can be used, for example, a method of utilizing tannic acid, formalin, glutaraldehyde, pyruvic aldehyde, bis-diazotized benzidine, toluene-2,4-diisocyanate, amino group, carboxyl group that can be converted into an active ester group, hydroxyl group or amino group that can be converted into phosphoramidite group, or the like.

[0244] The solid phase may be one usually used for immobilizing a protein, nucleic acid or the like, and material and shape thereof are not particularly limited. For example, glass plates, nitrocellulose membranes, nylon membranes, polyvinylidene fluoride membranes, microplates made of plastics and so forth can be used.

[0245] The "measurement" is a means for collecting changes of signals used for analysis, and it should not be construed in any limitative way. As the measurement method used, any of methods that can detect an intermolecular interaction can be used, including fluorescence correlation spectroscopy, fluorescence resonance energy transfer method, evanescent field molecular imaging method, fluorescence depolarization method, fluorescence imaging analysis method, surface plasmon resonance method, enzyme linked immunosorbent assay and so forth.

[0246] The measurement method includes a method comprising adding the modified protein of the present invention onto an array on which a target molecule is immobilized and detecting the modified protein of the present invention specifically binding to the target molecule. The array on which the target molecule is immobilized means a solid phase on which the target molecule is immobilized in an arrangement enabling identification thereof. The method for detecting the modified protein of the present invention specifically binding to the target molecule is not particularly limited, so long as the method enables detection of the modified

protein of the present invention specifically binding to the target molecule. However, there is usually used, for example, a method of removing the modified protein of the present invention not binding to the target molecule by washing from the array to which the modified protein of the present invention is added and detecting the remaining modified protein of the present invention.

[0247] Hereafter, examples of the measurement method will be explained.

[0248] (1) Fluorescence correlation spectroscopy

The fluorescence correlation spectroscopy (FCS, Eigen, M., et al., Proc. Natl. Acad. Sci., USA, 91, 5740-5747 (1994)) is a method of measuring flow rate, diffusion coefficient, volume shrinkage or the like of particles under a confocal laser microscope or the like. In the present invention, interacting molecules can be measured by measuring change of translational Brownian movement of one original modified molecule of the present invention (C-terminal modified protein) caused by an interaction between the modified protein and a target molecule.

[0249] Specifically, fluorescence emitted from sample particles in a partial volume of a sample solution due to excitation of the sample particles by an excitation light is measured to obtain a photon ratio. This value changes with the number of the particles existing in a space volume observed during a specific period of time. The aforementioned various parameters can be calculated from the change of signals using an autocorrelation function. Apparatuses for carrying out FCS are also marketed from Carl Zeiss and so forth, and analysis can be performed by using these apparatuses also in the present invention.

[0250] When a protein-target molecule interaction is measured or analyzed by using this method, it is required to provide both of the C-terminal modified protein and the target molecule as solutions (liquid phase method). The target molecule does not need to be labeled. Further, a molecule having a molecular weight extremely smaller than

that of the C-terminal modified protein of which interaction should be investigated is not suitable for this method, since such a molecule does not affect the Brownian movement of the C-terminal modified protein.

[0251] However, fluorescence cross-correlation spectroscopy (FCCS) using two kinds of fluorescent dyes can detect even an interaction between proteins having molecular weights of similar order, of which detection is difficult by FCS using one kind of fluorescent dye. Although the fluorescence resonance energy transfer (FRET) method is known as another method of using two kinds of fluorescent dyes, two kinds of fluorescent dyes need to approach each other at a distance within 40 to 50Å in order to cause FRET, and there is a risk in this method that FRET may not be observed depending on sizes of proteins, locations at which the fluorescent dyes are attached or the like, even an interaction occurs. On the other hand, since the detection of cross-correlation does not depend on the distance between the fluorescent dyes in the FCCS method, it does not suffer from such a problem. Further, comparing with the fluorescence depolarization method as another detection system, the FCCS method has advantages of a smaller amount of required sample, shorter detection time, easier automatization for HTS and so forth. Further, since the FCCS method provides extremely fundamental information such as size and number of fluorescence-labeled molecules, it may be used for general purpose like the surface plasmon resonance method. The difference between the both is that, in the surface plasmon resonance method, an interaction is detected in the state that proteins are immobilized, whereas the FCCS method enables observation of interaction in a solution, which is closer to a natural state. In the FCCS method, although proteins do not need to be immobilized, the proteins must be labeled with fluorescent dyes instead. However, it has been made possible by the present invention to overcome this problem.

[0252] Further, the FCCS method enables investigation

of a protein-protein interaction or protein-nucleic acid interaction in a state of solution, which is close to the intracellular environment, and enables convenient calculation of dissociation constant (binding constant) by one measurement.

[0253] The method for bringing a target molecule into contact with the C-terminal modified protein in this method may be any method that allows the contact in a sufficient degree such that they can interact with each other. However, it is preferably attained by a method of introducing a solution dissolving the C-terminal modified protein in a buffer usually used for biochemical purpose or the like at an appropriate concentration into a well for measurement in a commercially available FCS apparatus and further introducing a solution dissolving the target molecule in the same buffer at an appropriate concentration into the well.

[0254] In this method, as a method of performing multiple analyses, for example, there is used a method of introducing multiple kinds of different C-terminal modified proteins into wells for measurement in the aforementioned FCS apparatus, respectively, and further introducing a solution of a particular target molecule into the wells, or introducing a particular C-terminal modified protein into wells, and further introducing solutions of multiple kinds of different target molecules into the wells, respectively.

[0255] (2) Fluorescence imaging analysis method

The fluorescence imaging analysis method is a method of bringing a modifying molecule into contact with an immobilized molecule and measuring or analyzing fluorescence emitted by the immobilized modifying molecule remained on the immobilized molecule due to an interaction between the both molecules using a commercially available fluorescence imaging analyzer.

[0256] When a protein-target molecule interaction is measured or analyzed by using this method, one of the C-terminal modified protein or the target molecule must be

immobilized by the aforementioned method. When an immobilized target molecule is used, either a modified or unmodified target molecule can be used. Further, when it is used without immobilization, it must be modified with the aforementioned modification substance. Either a C-terminal modified protein immobilized at the modified portion or a C-terminal modified protein immobilized at a portion other than the modified portion may be used.

[0257] As a substrate for immobilizing a C-terminal modified protein or target molecule (solid phase), there can be used glass plates, nitrocellulose membranes, nylon membranes, microplates made of plastics and so forth, which are usually used for immobilizing a protein, nucleic acid or the like. Further, such substrates as mentioned above of which surfaces are bound with various functional groups (amino group, carboxyl group, thiol group, hydroxyl group etc.) or various ligands (biotin, iminobiotin, metal ions such as nickel or cobalt ion, glutathione, saccharides, nucleotides, DNA, RNA, antibody, calmodulin, receptor protein etc.) can also be used.

[0258] The method for bringing a modified target molecule or a C-terminal modified protein into contact with an immobilized molecule in this method may be any method that allows the contact in a sufficient degree such that the both molecules can interact with each other. However, it is preferably attained by a method of preparing a solution dissolving the modified target molecule or the C-terminal modified protein in a buffer usually used for biochemical purpose at an appropriate concentration and bringing the solution into contact with the solid phase surface.

[0259] After bringing the both molecules into contact with each other, a step of washing off excessively existing modified target molecule or C-terminal modified protein with the same buffer or the like is preferably performed, and fluorescence signal emitted from the modification substance of the target molecule or C-terminal modified

protein which remained on the solid phase, or a mixed signal of fluorescence emitted from the immobilized modified molecule and fluorescence emitted from the modified molecule remained on the solid phase can be measured or analyzed by using a commercially available imaging analyzer to identify the molecule that interacts with the immobilized molecule.

[0260] In this method, as a method of simultaneously performing multiple analyses, for example, there is used a method of immobilizing multiple kinds of C-terminal modified proteins or modified or unmodified target molecules on the aforementioned solid phase surface with positioning addresses, a method of bringing multiple kinds of non-immobilized C-terminal modified proteins or modified target molecules into contact with one kind of C-terminal modified protein or modified or unmodified target molecule, or the like. When multiple kinds of C-terminal modified proteins or modified target molecules are brought into contact, the molecules remained on the solid phase can be obtained by dissociating them using difference of buffer concentration or the like and analyzed by a known method to identify them.

[0261] (3) Fluorescence resonance energy transfer method

As another intermolecular interaction detection method using two kinds of fluorescent dyes, the fluorescence resonance energy transfer (FRET) method is well known. FRET means a phenomenon that, if a fluorescence spectrum of one of two kinds of fluorescent dyes (energy donor) and an absorption spectrum of the other (energy receptor) overlap, and the distance between two of the fluorescent dyes is sufficiently small, it becomes more likely that excitation energy of the donor excites the receptor before the donor emits fluorescence. Therefore, two kinds of proteins of which interaction is desired to be detected are labeled with fluorescent dyes serving as the donor and the receptor, respectively, and the donor is excited. When the two kinds of proteins do not interact

with each other, FRET is not caused because the distance between the fluorescence dyes is large, and thus fluorescence spectrum of the donor is observed. However, if the two kinds of proteins interact with each other, and hence the distance between the fluorescent dyes becomes smaller, fluorescence spectrum of the receptor is observed due to FRET. Therefore, presence or absence of an interaction between the proteins can be determined on the basis of difference in wavelengths of fluorescence spectra. As for the fluorescent dyes, a combination of fluorescein as the donor and rhodamine as the receptor is frequently used. Further, it is recently attempted to observe FRET in a cell to detect an interaction by using combination of mutant green fluorescence proteins (GFP) emitting fluorescence of different wavelengths. As a drawback of this method, it is mentioned that since two kinds of fluorescent dyes need to approach to each other at a distance within 40 to 50Å in order to cause FRET, there is a risk that FRET may not be observed depending on sizes of proteins, locations at which the fluorescent dyes are attached or the like, even if an interaction occurs.

[0262] (4) Evanescent field molecular imaging method

The evanescent field molecular imaging method is a method described in Funatsu, T., et al., Nature, 374, 555-559 (1995) or the like, and it is a method of bringing a second molecule as a solution into contact with a molecule immobilized on a transparent material such as glass, irradiating them with a laser light or the like from a light source at such an angle that an evanescent field should be generated, and measuring or analyzing the generated evanescent light using a detector. These operations can be performed by using an evanescent field fluorescence microscope known per se.

[0263] When a protein-target molecule interaction is measured or analyzed by using this method, one of the C-terminal modified protein or the target molecule must be immobilized by the aforementioned method. When an

immobilized target molecule is used, it does not need to be modified. However, when it is used without immobilization, it must be modified with the aforementioned modification substance.

[0264] As the substrate for immobilizing the C-terminal modified protein or target molecule, a substrate made of a material of glass or the like is used, and quartz glass is preferably used. Further, a substrate of which surface is cleaned by ultrasonication is preferred in order to prevent scatter of laser light or the like.

[0265] The method for bringing a non-immobilized C-terminal modified protein or target molecule into contact with an immobilized molecule in this method may be any method that allows the contact in a sufficient degree such that the both molecules can interact with each other. However, a method of preparing a solution dissolving the non-immobilized C-terminal modified protein or modified target molecule in a buffer usually used for biochemical purpose at an appropriate concentration and adding the solution dropwise to the solid phase surface is preferred.

[0266] After bringing the both molecules into contact with each other, fluorescence generated through excitation by the evanescent field illumination can be measured by using a detector such as a CCD camera to identify the molecule that interacts with the immobilized molecule.

[0267] In this method, as a method of simultaneously performing multiple analyses, for example, there is used a method of immobilizing multiple kinds of C-terminal modified proteins or modified target molecules on the aforementioned substrate with positioning addresses, or the like.

[0268] (5) Fluorescence depolarization method

The fluorescence polarization method (Perran, J., et al., J. Phys. Rad., 1, 390-401 (1926)) is a method utilizing the fact that a fluorescent molecule excited with a polarized fluorescent light emits fluorescence in the same plane of polarization during the excited state while

it maintains a stationary state, whereas the emitted fluorescence has a plane different from that of the excitation light when the excited molecule undergoes rotational Brownian movement or the like during the excited state. The movement of molecule is affected by the size thereof, and when the fluorescent molecule is a macromolecule, the molecule scarcely shows movement during the excited state, and emitted light is maintained to be a polarized light. However, in the case of a low molecular weight fluorescent molecule, since it shows high moving velocity, the emitted light is depolarized. Therefore, if intensity of the fluorescence emitted from a fluorescent molecule excited by a plane polarized light is measured along the original plane and a plane perpendicular thereto, information of motility and existing state of the molecule can be obtained from a ratio of the fluorescence intensities for the both planes. According to this method, behavior of a target molecule that interacts with a fluorescence-modified molecule can be traced without being affected by contaminants, if any. This is because shift of polarization degree is measured only when the fluorescence-modified molecule and the target molecule interact with each other.

[0269] As apparatuses for carrying out this method, BECON (produced by Panyera) and so forth are marketed, and this method can be carried out by using these apparatuses.

[0270] When a protein-target molecule interaction is measured or analyzed by using this method, it is required to provide both of the C-terminal modified protein and the target molecule as solutions. The target molecule does not need to be modified. Further, a molecule having a molecular weight extremely smaller than that of the C-terminal modified protein of which interaction should be investigated is not suitable for this method, since such a molecule does not affect the Brownian movement of the C-terminal modified protein.

[0271] The method for bringing a target molecule into

contact with the C-terminal modified protein in this method may be any method that allows the contact in sufficient degree such that they should interact with each other. However, it is preferably attained by a method of introducing a solution dissolving the C-terminal modified protein in a buffer usually used for biochemical purpose at an appropriate concentration into a well for measurement in a commercially available fluorescence depolarization apparatus and further introducing a solution dissolving the target molecule in the same buffer at an appropriate concentration into the well.

[0272] It is expected that specificity of interaction between the C-terminal modified protein and the target molecules to be measured in this method is not necessarily so high as that of an antigen-antibody reaction. Therefore, in order to identify an optimum combination, it is effective that degree of interaction should be numerically defined. As an index representing degree of interaction, for example, a value of the minimum target substance concentration providing the maximum fluorescence polarization degree for a C-terminal modified protein of a fixed concentration or the like can be used.

[0273] In this method, as a method of simultaneously performing multiple analyses, for example, there is used a method of introducing multiple kinds of different C-terminal modified proteins into wells for measurement in the aforementioned fluorescence depolarization apparatus, respectively, and further introducing a solution of a particular target molecule into the wells, or introducing a particular C-terminal modified protein into wells and further introducing solutions of multiple kinds of different target molecules into the wells, respectively.

[0274] (6) Surface plasmon resonance method

The surface plasmon resonance method is a method of measuring surface plasmon excited by a molecule interacting at a metal/liquid interface as change of intensity of reflected light (Cullen, D.C., et al., Biosensors, 3 (4),

211-225 (1987-88)). When a protein-target molecule interaction is measured or analyzed by using this method, the C-terminal modified protein must be immobilized by the aforementioned method, but the target molecule does not need to be modified.

[0275] As a substrate for immobilizing the C-terminal modified protein, a transparent substrate made of glass or the like on which a thin film of metal such as gold, silver or platinum is formed is used. The transparent substrate may be any of those usually used for surface plasmon resonance apparatuses. It generally consists of glass as a substrate consisting of a material transparent to a laser light, and such a substrate having a thickness of about 0.1 to 5 mm is generally used. Further, thickness of the metal thin film is suitably about 100 to 2000 Å. Those marketed as such immobilization substrates for surface plasmon resonance apparatuses can also be used. The C-terminal modified protein can be immobilized on the substrate by the method described above.

[0276] The method for bringing a target molecule into contact with the C-terminal modified protein in this method may be any method that allows the contact in a sufficient degree such that the both molecules can interact with each other. However, a method of bringing the immobilized C-terminal modified protein into contact with a solution dissolving the target molecule in a buffer usually used for biochemical purpose at an appropriate concentration can be preferably used.

[0277] These steps may also be performed by using a commercially available surface plasmon resonance apparatus, for example, BIAcore 2000 (produced by Pharmacia Biosensor). After bringing the both molecules into contact with each other, change with time of relative intensity of each reflected light can be measured by using a surface plasmon resonance apparatus known per se to analyze or measure an interaction of the immobilized C-terminal modified protein and the target molecule.

[0278] In this method, as a method of simultaneously performing multiple analyses, for example, there is used a method of immobilizing multiple kinds of C-terminal modified proteins on a substrate used for the surface plasmon resonance apparatus with positioning addresses, a method of bringing multiple kinds of target molecules into contact with one kind of immobilized C-terminal modified protein, or the like.

[0279] (7) Enzyme linked immunosorbent assay

The enzyme linked immunosorbent assay (ELISA, Crowther, J.R., Methods in Molecular Biology, 42 (1995)) is a method of bringing a solution containing an antibody into contact with an antigen immobilized on a solid phase and measuring or analyzing the antibody remaining on the immobilized antigen due to the interaction between the both molecules (antigen-antibody reaction) on the basis of fluorescence emitted from a modification molecule (IgG etc.) specifically binding to the antibody or a signal emitted by a dye formed from the modification molecule as a substrate using a commercially available detector (ELISA reader).

[0280] When a protein-target molecule interaction is measured or analyzed by using this method, the C-terminal modified protein serving as the antigen must be immobilized by the aforementioned method. Further, the target molecule serving as the antibody must be modified with the aforementioned modification substance.

[0281] As a substrate for immobilizing the C-terminal modified protein serving as the antigen, microplates made of plastics usually used for ELISA and so forth can also be used.

[0282] The method for bringing the modified target molecule serving as the antibody into contact with an immobilized molecule in this method may be any method that allows the contact in a sufficient degree such that the both molecules can interact with each other. However, a method of preparing a solution dissolving the modified

target molecule in a buffer usually used for biochemical purpose at an appropriate concentration and introducing the solution into a microplate is preferred.

[0283] After bringing the both molecules into contact with each other, a step of washing off excessively existing modified molecule not binding to the immobilized molecule is preferably performed, and fluorescence emitted from the modified molecule remained on the solid phase can be measured or analyzed by using a commercially available ELISA reader or the like to identify the molecule that interacts with the immobilized antigen molecule.

[0284] In this method, as a method of simultaneously performing multiple analyses, for example, there is used a method of immobilizing multiple kinds of different modified target molecules in each well of the aforementioned microplate.

[0285] Further, the protein of the present invention can also be used for identification of a molecule that causes an interaction.

[0286] When primary structure of a target molecule for which an interaction with a C-terminal modified protein is recognized on the basis of measurement according to any one of the methods described above is unknown, the primary structure can be analyzed by a suitable method known per se. Specifically, when the target molecule for which an interaction is recognized is a protein, its amino acid sequence can be analyzed by using an amino acid analyzer etc. to identify the primary structure. Further, when the target molecule is a nucleic acid, nucleotide sequence can be determined by a nucleotide sequence determination method using an automatic DNA sequencer or the like.

[0287] Furthermore, the protein of the present invention can also be used for analysis of an interaction with a protein library.

[0288] The present invention provides a gene or nucleic acid sequence encoding a novel protein that can form a complex with the c-Jun protein, which was obtained

by performing cotranslation selection/screening of IVV using the c-Jun protein as the bait and a mouse brain cDNA library as the prey, and methods for utilizing them. The present invention also provides a method for utilizing a gene or nucleic acid sequence encoding a known protein, which is not known to form a complex with the c-Jun protein.

[0289] The present invention not only enables screening of known gene sequences and known nucleic acid sequences, but also can provide a novel protein having a novel amino acid sequence formed by unexpected frame shift, a novel protein having a nucleic acid sequence for which only the nucleic acid sequence is published on the basis of genome information, or a novel protein having a completely novel nucleic acid sequence, further, a protein that forms a complex by an unexpected indirect interaction in addition to a direct interaction, a gene or nucleic acid sequence encoding the protein, and methods for utilizing them.

[0290] Hereafter, the amino acid sequences of the proteins of the present invention and the sequences of the nucleic acids encoding them will be specifically described. However, the following examples should be construed as a mere aid for specifically understanding the present invention, and the scope of the present invention is no way limited by the following examples.

Example 1

[0291] Cotranslation selection/screening of IVV was carried out by using the c-Jun protein as a bait and a mouse brain cDNA library as a prey (Fig. 2), and as a result, genes or nucleic acid sequences encoding novel proteins that can form a complex with the c-Jun protein were obtained.

[0292] The preparation method of the bait, c-Jun protein, was as follows. A DNA template was prepared from a pCMV-cJun(502-957)CBPzz vector (SEQ ID NO: 256) by PCR (primers 5' SP6(029)T7 (SEQ ID NO: 257) and 3' FosCBPzz (SEQ ID NO: 258), and PCR program CYCB1 (refer to Table 1))

using TaKaRa Ex Taq (Takara Shuzo). The DNA template was transcribed (37°C, 2 hours) by using RiboMAX™ Large Scale RNA Production Systems (Promega) to prepare a mRNA template of the bait c-Jun protein. A bait DNA made to coexist was prepared by PCR (primers 5' DNA (SEQ ID NO: 260) and 3' DNA (SEQ ID NO: 161)) using DNA-Fos/Jun (SEQ ID NO: 259) containing the Fos/Jun binding sequence as a template according to the PCR program V-2 (refer to Table 1).

[0293] The preparation method of the mouse brain cDNA library as the prey was as follows. An IVV random library was prepared as shown in Fig. 3. As an RNA library, a commercially available mouse brain (polyA+) RNA library (obtained by purifying a tissue extracted RNA library in an oligo dT column, Clontech) was purchased. As for design of an adaptor, it was designed so as to add a 5' UTR sequence suitable for the production of assigning molecules (promoter SP6 + enhancer O29 or O') to the library as a sequence required for IVV formation. For the mouse brain (polyA+) RNA library, an adaptor having the enhancer O29 was used. The main chain (SEQ ID NO: 262 or 263) and the subchain (gaattcgc) of the adaptor for the enhancer O29 were each dissolved in the TE buffer (10 mM Tris-Cl, pH8.0, 1 mM EDTA) at a concentration of 100 µM, and 10 µl each of the solutions of the main chain and subchain were mixed so that the main chain and the subchain were mixed in equimolar amounts. The mixture was heated at 90°C for 2 minutes and at 70°C for 5 minutes, set on a water bath of 60°C, and then slowly cooled from 60°C to room temperature by turning off the heater of the bath. The mixture was divided into 5 µl aliquots, and stored at -20°C. Then, the mouse brain (polyA+) RNA library was reverse-transcribed into single stranded DNAs (Fig. 3, I). 0.5 µg of the mouse brain (polyA+) RNA library (1.4 pmole/0.5 µg), 2 pmol of 3' random primer (SEQ ID NO: 264) and DEPC water were added to obtain a volume of 12.0 µl, and the mixture was heated at 70°C for 10 minutes, and cooled for 1 minute on ice. A reverse transcription reaction was performed at 45°C for 1

hour by using this mixture and SuperScriptII RT (SuperScript Double-stranded cDNA Synthesis Kit, Invitrogen). Then, the total amount of the single stranded DNAs synthesized by the reverse transcription reaction was used for a reaction with an *E. coli* DNA ligase, *E. coli* Polymerase I and *E. coli* RNase H (SuperScript Double-stranded cDNA Synthesis Kit, Invitrogen) at 16°C for 2 hours, and the product was blunt-ended with T4 DNA polymerase at 16°C for 5 minutes to synthesize double-stranded DNAs (Fig. 3, II). Then, the adaptor previously prepared was ligated by taking advantage of the fact that the 5' ends of the double-stranded DNAs were phosphorylated (Fig. 3, III). The synthesized double-stranded DNA library was subjected to ethanol precipitation, and dissolved in 4 µl of DEPC water. 100 µM of the prepared adaptor in a volume of 1.0 µl and 50 µl of Ligation High (TOYOBO) were added thereto, reacted overnight at 16°C, purified (DNA purification kit, QIAGEN), and then adjusted to a volume of 50 µl. Thereafter, PCR (EX Taq Hot Start Version, TaKaRa) was performed (Fig. 3, IV). Out of 50 µl of the ligated double-stranded DNA library, 2 µl was used as a template together with 5' PCR primer (SEQ ID NO: 260) having a specific sequence required for IVV (O29) and 3' PCR primer (SEQ ID NO: 261) to prepare an IVV cDNA library. As for the PCR conditions, the total volume was 100 µl, and 22 cycles of the reactions (each cycle consists of reactions at 94°C for 30 seconds, at 60°C for 30 seconds, and at 72°C for 90 seconds, and the final extension reaction was performed at 72°C for 180 seconds).

[0294] Cotranslation (26°C, 60 minutes) of the mRNA template of the bait c-Jun protein, the mouse brain cDNA library as the prey, and the bait DNA made to coexist was carried out in a cell-free translation system of wheat (Wheat Germ Extract, Promega) in a volume of 50 µl. To 50 µl of the sample, 50 µl of IgG binding buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% NP40) was added to obtain the total volume of 100 µl (cotranslation sample). Then, IgG

agarose (Sigma) was washed twice with the IgG binding buffer, and the cotranslation sample (100 μ l) was added thereto, and the mixture was stirred by rotation at 4°C for 2 hours. The IgG agarose was washed 3 times with the binding buffer and once with a TEV cleaving buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT), and the bait/prey complex binding to the IgG agarose was cleaved with TEV protease (GIBCO-BRL, 16°C, 2 hours). By using 1 μ l of the collected 100 μ l solution as a template, RT-PCR (One step RT-PCR kit (QIAGEN), primers: SEQ ID NOS: 265 and 266, program: RT-QH3090 (refer to Table 1)) was carried out in a volume of 50 μ l. After this procedure (Step 1 in Fig. 2) was repeated for 5 rounds, the library was cloned and sequenced to obtain the amino acid and nucleotide sequences of the genes shown in Fig. 1. The DNA sequence SEQ ID NOS. corresponding to the amino acid SEQ ID NOS.: 1 to 69 are shown in List 1 below. The same results were obtained for both of the library prepared by using the sequence of SEQ ID NO: 262 as the main chain of the adaptor for enhancer O29 and the library prepared by using the sequence of SEQ ID NO: 263 as the same.

[0295] <List 1>

126(1), 127(2), 128(3), 129(4), 130(5), 131(6), 132(7),
 133(8), 134(9), 135(10), 136(11), 137(12), 138(13), 139(14),
 140(15), 141(16), 142(17), 143(18), 144(19), 145(20),
 146(21-1), 147(21-2), 148(22), 149(23), 150(24), 151(25),
 152(26), 153(27), 154(28), 155(29), 156(30), 157(31),
 158(32), 159(33), 160(34-1), 161(34-2), 162(35), 163(36),
 164(37), 165(38), 166(39), 167(40), 168(41), 169(42),
 170(43-1), 171(43-2), 172(44), 173(45), 174(46), 175(47),
 176(48), 177(49), 178(50), 179(51), 180(52), 181(53),
 182(54), 183(55), 184(56), 185(57), 186(58), 187(59),
 188(60), 189(61-1), 190(61-2), 191(62), 192(63), 193(64-1),
 194(64-2), 195(65), 196(66), 197(67), 198(68), 199(69)

[0296] All the proteins are proteins found by the present invention for the first time to form a complex with c-Jun.

[0297] As a verification experiment of the interactions of the obtained proteins and c-Fos, expression of the proteins of SEQ ID NOS: 18 (SNAP19), 76 (KINN), 93 (Kif5a), 99 (Eef1d), 102 (Nef3), 106 (Jip-c3.1), 110 (Jip-cl), 113 (EB2), 115 (Cspg6), 117 (Mapk8ip3), 119 (Jip-c3.2), 121 (GFAP), 123 (Jip-c8) and 125 (Kif5b) (Fig. 1) in a cell-free translation system was experimentally confirmed by using the DNA sequences of SEQ ID NOS: 143, 206, 223, 229, 232, 236, 240, 243, 245, 247, 249 and 253 according to the descriptions of WO02/46395, Example 1, (2) Preparation of coding molecule and (3) Translation of coding molecule, i.e., it was confirmed by the C-terminal labeling method that the proteins were expressed in a wheat cell-free translation system (Fig. 4, A). Further, the C-terminal labeled proteins for which expression was confirmed, were used as a prey protein to confirm interactions thereof with the bait c-Jun by pull-down. As for the preparation method of the prey protein, specifically, PCR cloning kit (QIAGEN) was used to extract a sequence cloned in the pDrive vector (SEQ ID NO: 267, QIAGEN) from cells, and a DNA template was prepared by PCR (primers 5'F3 (SEQ ID NO: 268) and 3'R3 (SEQ ID NO: 269), PCR program: ISHI1562 (refer to Table 1), 100 µl scale) using TaKaRa Ex Taq (Takara Shuzo). The DNA template was transcribed (37°C, 2 hours, 50 µl scale) by using RiboMAX™ Large Scale RNA Production Systems (Promega) to prepare a mRNA template of the prey protein.

[0298] The preparation method of the bait c-Jun protein is the same as that used for the screening.

[0299] Cell-free translation of the prey template (10 µl scale) was performed for 1 hour by using the C-terminal labeling method to prepare a prey protein in a C-terminal labeled state. At the same time, the translation reaction of the bait c-jun template was performed for 1 hour by the cell-free translation (50 µl scale) to produce the bait protein. After the translation, the both and the binding buffer were mixed (prey: 8 µl, bait: 10 µl, IgG binding buffer: 82 µl), and incubated with 50 µl of IgG agarose

beads for 2 hours, and the beads were washed, then added with 20 μ l of a buffer containing SDS, boiled at 100°C for 5 minutes, and eluted. This sample was developed by 17.5% SDS-PAGE, and the FITC fluorochrome was observed by means of a fluorescence imager (Fig. 4, B). In addition, a reaction was also performed without adding the bait c-Jun protein as a control.

[0300] As a result, it could be confirmed that the proteins of SEQ ID NOS: 2 (SNAP19), 71 (KINN), 89((Kif5a), 96 (Eef1d), 101((Nef3), 106 (Jip-c3.1), 110 (Jip-c1), 113 (EB2), 115 (Cspg6), 117 (Mapk8ip3), 119 (Jip-c3.2) and 123 (Jip-c8) directly interacted with c-Jun.

[0301] Furthermore, concentration of genes directly or indirectly interacting with c-Jun was confirmed by real time PCR using the nucleic acid sequences of SEQ ID NOS: 143, 206, 223, 229, 232, 236, 240, 243, 245, 247, 249, 253 and 255 (Fig. 5). Because SEQ ID NOS: 251 (GFAP) and 255 (Kif5b) were also concentrated similarly to other genes of direct interaction, it could be confirmed that there were indirect interaction with c-Jun. As for the specific method of the real time PCR, primers (SEQ ID NOS: 270 to 297) were designed for fourteen kinds of genes (SEQ ID NOS: 143, 206, 223, 229, 232, 236, 240, 243, 245, 247, 249, 253 and 255) so that the amplification should be attained in the ranges of sequences obtained by the screening. For preparation of calibration curves, a gene comprising a DNA fragment of positive control incorporated into the pDrive vector was amplified by PCR (5'M13_F primer (SEQ ID NO: 298) and 3'M13_R primer (SEQ ID NO: 299) were used, and the PCR program lightcycler of Table 1 was used), which was controlled so that 1E03, 1E05, 1E07 or 1E09 clones/reaction should be obtained. The measurement was controlled so that each of the library DNA before screening, library DNA in each cycle of screening, and Mock library DNA not added with the bait c-jun should be in an amount of 5 ng/reaction. The PCR measurement reaction was performed in a scale of 20 μ l according to the programs shown in Table 1 by using

LightCycler Instrument or LightCycler FastStart DNA Master SYBR Green I (both are produced by Roche Diagnostics).

[0302] Further, the proteins and genes or nucleic acid sequences of the present invention can be used as an inhibitor for blocking transcription, gene duplication and so forth as functions of c-Jun by utilizing the novel function thereof (function of enabling binding with c-Jun in this case). The basis of the above is originates in the fact that the genes detected by the IVV method have been detected through a competitive process constituted by screening repeated multiple times. Therefore, the genes detected by the IVV method show a certain number distribution, and a gene having a stronger competitive power should be detected in a larger number. This suggests that a larger number of clones corresponds to stronger competitive power, and thus such a gene acts more effectively as a blocking agent or inhibitor. In the IVV selection performed in this example, three (/132) of c-Fos well known as a prey, one (/132) of c-Jun, one (/132) of ATF4 and five (/132) of Jpp2 were detected for the bait c-Jun. Thus, the numbers of clones (Fig. 1) detected in the selection indicate that SNAP19, KINN and so forth have extremely stronger competitive power compared with known proteins; Kif5a, Eef1d, Nef3, Jip-c3.1, Jip-c1 and so forth can sufficiently compete with c-Fos; and EB2, Cspg6, Mapk8ip3, Jip-c3.2, Jip-c8 and so forth can sufficiently compete with c-Jun or ATF4, and thus the proteins can be utilized as an inhibitor for blocking functions of transcription of a complex, gene duplication and so forth by the interaction of c-Jun and a known protein.

[0303]

Table 1: PCR programs

Program name: CYCB1

Reaction conditions:

95°C	1 minute		
98°C	20 seconds	←	15 cycles
55°C	1 minute		
72°C	4 minutes		
4°C	Pause		

Program name: V-2

Reaction conditions:

98°C	20 seconds	←	35 cycles
55°C	1 minute		
72°C	3 minutes		
4°C	Pause		

Program name: RT-QH3090

Reaction conditions:

50°C	30 minutes		
95°C	15 minutes		
94°C	30 seconds	←	(30 cycles for 1st round, 26 cycles for 2rd to 4th rounds 28 cycles for 5th round)
60°C	30 seconds		
72°C	90 seconds		
72°C	10 minutes		

Program name: ISHI1562

Reaction conditions:

94°C	2 minutes		
94°C	30 seconds	←	15 cycles
62°C	30 seconds		
73°C	2 minutes		
73°C	15 minutes		

Program name: lightcycler

Reaction condition:

95°C	10 minutes		
95°C	15 seconds	←	40 cycles
X°C	10 seconds		
72°C	5 seconds		

X: Annealing temperature was 62 to 51°C depending on the T_m values of primers.

Industrial Applicability

[0304] Because proteins that interact with c-Jun have been provided, it becomes possible to provide proteins forming a complex with c-Jun by not only a direct interaction, but also an unexpected indirect interaction, and nucleic acids encoding these proteins as well as methods for utilizing them.